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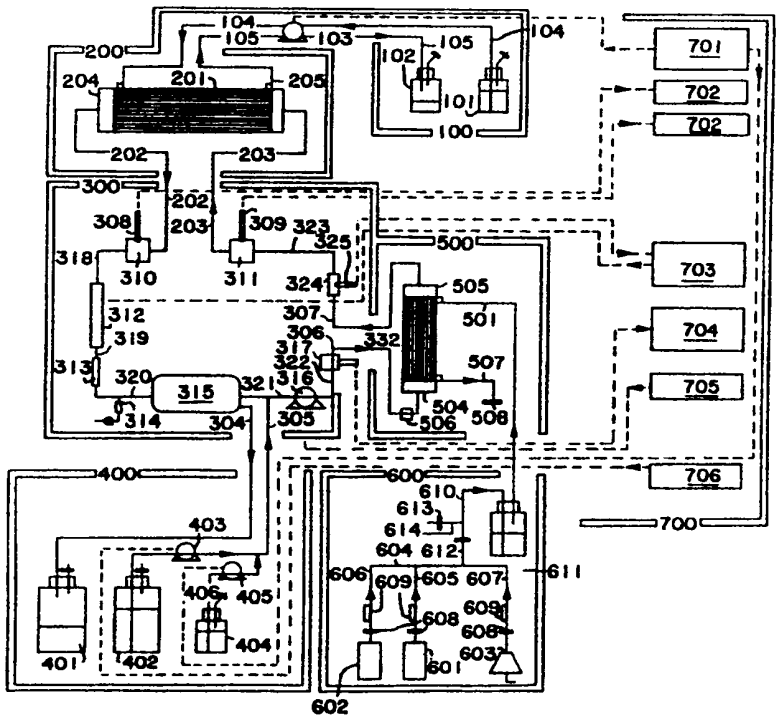

(57) Abstract

A hollow fiber bioreactor system and method for the propagation of cells and the production of various cell propagation products is described. The system uses at least two hollow fiber bioreactors (201) and interrelated detection and regulation of dissolved oxyg n (502, 504), basal medium (706) and reagent composition (706, 701, 403), temperature (312, 325, 703), pH, and waste disposal (806, 808, 805).

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HOLLOW FIBER CELL PROPAGATION

This is a continuation-in-part application of application Serial No. 07/480,533 filed 17 February 1990 and a continuation of application Serial No. _____, filed 12 February 1991.

FIELD OF THE INVENTION

This invention relates to a system and a method for the propagation of cells and to the production of various cell propagation products. More particularly particularly, the invention relates to the propagation of cells in a unique hollow fiber bioreactor system and process.

BACKGROUND OF THE INVENTION

The propagation of suspension and anchorage dependent cells in hollow fiber bioreactors is variously described in the prior art. In general, known procedures entail the use of bioreactors comprising a plurality of media permeable parallel hollow fibers surrounded by an extracapillary space (ECS). Cell growth medium passed through the hollow fiber lumens permeates the lumen walls to support cell growth in the ECS. See, e.g. U.S. Patents 3,821,087; 4,439,322 and Ramsay et al. In Vitro 20:10 (1984).

SUMMARY OF THE INVENTION

This invention provides a unique hollow fiber cell propagation system and process. The cell propagation system is compact and highly efficient. It includes a plurality of novel elements including a bioreactor, or bioreactors, having hollow fibers of unique composition and interrelated means to control dissolved oxygen, basal medium and reagent composition, temperature, pH and waste disposal means.

The process as practiced in the system of the invention provides high cell and product yields with a low consumption of medium and nutrients. These benefits ar attributable to a plurality of innovations which individually and in combination provide for the uniquely efficient propagation of suspension and anchorage dependent mammalian, insect, avian, plant and fungal cells and associated products.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration of the cell propagation system of the invention.

Figure 2 is a sectional view of a dissolved oxygen probe and probe block useful in the system of the invention.

Figure 3 is a schematic view of a means, useful in the invention for control of the temperature of the basal medium and reagents utilized in the system of the invention.

Figure 4 is a schematic view of a means useful to control the composition of the basal medium and reagents utilized in the system.

Figure 5 is a schematic view of a waste disposal system useful in the invention.

Figure 6 is a schematic view of an oxygenator useful in the system of the invention.

Figure 7 is a sectional view of a loop heater useful in the system of the invention.

Figure 8 is a schematic illustration of an integrated, compact configuration of a single bioreactor system which embodies the invention; elements shown in phantom by broken lines are positioned behind elements shown in solid lines; all elements are directly or indirectly mounted on a transparent polycarbonate mounting sheet.

Figure 9 is a graph which illustrates the parallelism between GUR and ΔDO in a relatively aerobic system run.

Figure 10 is a graph which illustrates the gradual decrease in aerobic efficiency of a bioreactor during a 110 day system run.

Figure 11 is a schematic view of a multiple bioreactor recirculation loop configuration.

Figure 12 is a schematic view of a multiple bioreactor showing an extracapillary space nutrient delivery and harvest configuration.

DETAILED DESCRIPTION OF THE INVENTION

In the specification, the following symbols have the indicated significance or meaning:

DO1	Dissolved oxygen value upstream of bioreactor(s), expressed as % (percentage of air saturation)
DO2	Dissolved oxygen value downstream of bioreactor(s), expressed as % (percentage of air saturation)
ΔDO	Dissolved oxygen differential: $\Delta DO = DO1 - DO2$
ECS	Extracapillary space
Gi	Glucose concentration entering system, expressed in mg/l
Go	Glucose concentration exiting system, expressed in mg/l
GUR	Glucose utilization rate, expressed in mg/hr Calculated as: $GUR = \frac{v(Gi-Go)}{1000}$ where v = basal medium feed rate
Basal Medium Feed Rate	Rate at which basal medium and reagents enter and leave the system. Expressed in ml/hr
Lumen (or Loop)	Space inside a hollow fiber
Basal Medium	Nutrient medium comprises water, salts, amino acids, vitamins.
Reagents	Substances added to a medium and known to have a particular action under certain conditions

Plateau Section	Portion of hollow fiber run at which process conditions are stable
Growth Factor Mixture	Combination of defined basal medium growth factors and/or serum
System Cancel-lation	Time at which growth support for the hollow fiber system is terminated

In general, the cell propagation process of the invention may include some or all of the steps 1 to 14 described hereinafter.

1. Cell lines are grown in several different basal medium and reagent types and combinations in order to select the most appropriate growth medium for the hollow fiber run. The selected growth medium is reduced to the simplest practical form.

2. Following hollow fiber system sterilization and assembly, basal medium is fed into the system and temperature control is set at about 37° Celsius.

3. Target Go values, preferably in a range of 50-500 mg/l and GUR (range is product and cell line dependent) values are selected for the plateau section of the run.

4. Gas composition (CO_2, N_2, air) is selected for system start up based upon pH requirements and cell line sensitivity to O_2. These gases are blended and humidified to approximately 100% relative humidity before delivery to the oxygenator.

5. Loop circulation rates are established which will support bioreactor oxygen demands generated by targeted GURs. DO2 values are preferably held above 10% saturation of air by lumen flow selection.

6. ECS growth factor mixture is formulated based upon historical experimentation. ECS growth factor delivery and product harvest program is developed as

a function of targeted GUR, feedback inhibition data, cell line and product requirements. ECS growth factor delivery and product harvest program is entered into the programmable controller.

7. Basal medium feed rate is set by adjusting the basal medium feed pump. The initial feed rate may be set high (in excess of that required for the targeted GUR) to provide accelerated generation of cell mass.

8. A cell inoculum is introduced into the ECS of the bioreactor(s). Cell count of the inoculum is cell line and bioreactor surface area dependent.

9. As target Go and GUR are approached, basal medium feed rates are reduced step-wise until target Go and GUR values are reached substantially simultaneously.

10. CO_2 flows are gradually reduced as target GURs are reached to maintain pH values in preferred ranges.

11. Productivity (protein production) is correlated with ΔDO values.

12. GURs are held constant by fine adjustments in basal medium to feed rate and system reagent composition.

13. Aerobic efficiency of the system is monitored by the ratio of GUR/ΔDO and as it relates to product output. System cancellation is determined by comparing these values to established limits developed through production cost analysis.

14. Data generated by the run may be added to the general data base and used to further define parameters such as optimal operational GURs, ΔDOs, ECS programming (growth factor delivery frequency, product harvest frequency and volume) and reagent recipes in future runs.

The system in which this process may be practiced is schematically illustrated by Figures 1 to 7 and 11 to 12.

THE SYSTEM OF THE INVENTION

As shown in Figure 1, the system includes interconnected ECS product/feed section 100, bioreactor section 200, loop section 300, loop feed/waste section 400, oxygenator section 500, gases section 600 and electronics section 700. The system preferably takes the compact form shown by Figure 8 in which various of the system elements are mounted on a plastic, preferably transparent polycarbonate mounting sheet. As shown in Figure 1, the bioreactor section 200 includes one bioreactor 201. This arrangement can be expanded with multiple bioreactors 201 as shown in Figures 11 and 12.

The ECS Product/Feed Section

The ECS product/feed section 100 includes an ECS feed reservoir 101, an ECS product reservoir 102, and an ECS pump 103. ECS feed reservoir 101 is connected by lines 104 to the pump 103, and the bioreactor section 200. ECS product is connected by lines 105 to the pump 103 and the bioreactor section 200.

The BioReactor Section 200

As shown in Figure 1, the bioreactor section 200 includes one hollow fiber bioreactor 201 connected through lines 104 and 105 to the ECS product/feed section 100 and through lines 202 and 203 to the loop section 300. The bioreactor 201 is provided at each end with manifolds 204 and 205 to which lines 202 and 203 are connected.

In order to increase cell production hollow fiber surface area can be increased by adding bioreactors to the system. Increasing the number of bioreactors,

however, results in proportional increases in system complexity and a tendency to lose cell mass homogenity because of unequal medium flow in both the ECSs and lumens of various bioreactors. Uneven medium flows cause not only unequal distribution of growth nutrients but also unequal product harvesting. These problems are overcome by the multiple bioreactor section of the present invention through use of unique configurations for medium flow paths and employment of unique system components. Further, the system configuration of the present invention is not limited to either an even or odd number of bioreactors, but can be applied to various numbers of bioreactors in parallel.

The recirculation loop configuration of the present invention for multiple bioreactors is shown in Figure 11. This recirculation loop configuration consists of a manifold 211 which is designed to split flows from a single loop stream, i.e., lines 202 and 203, into multiple loop streams required in such a number as to service multiple bioreactors 201 arranged in parallel with respect to each other. The manifold 211 is configured such that a single stream enters and a single stream exits. The single entrance stream and single exit stream provide fluid flow at diagonally opposite points to the manifold 211. Such an arrangement of equal size bioreactors 201 promotes nearly equal flow rates through lumens of each bioreactor 201 and therefore promotes equal metabolic activity in each bioreactor 201 which in turn results in equal product formation in each bioreactor 201. As with the single bioreactor 201 system shown in Figure 1, dissolved oxygen

measurements are made upstream of line 203 and downstream of line 202 in the loop section 300 described below.

Designs as described for loop flow manifolds 211 for multiple bioreactors 201 have little utility for the ECS flow system for multiple bioreactors 201 due to low flow rates and also low ECS pressures which create poor flow distribution patterns. A unique non-invasive pinch valve system and manifold is used in the present invention to effectively isolate each bioreactor 201 in the parallel configuration so that delivery of growth factor mixture and harvesting of ECS product is accurate in volume and specific for each bioreactor 201. Interreactor 201 flow is prevented on the upstream side by the presence of ECS pinch valves and on the downstream side by the presence of equal transmembrane pressures in each bioreactor 201.

A programmable controller 701, as shown in Figure 12, operates through the broken lines on ECS pump 103 in conjunction with a battery of pinch valves 210, 212, 214 and 216 at a desired frequency. The ECS pump 103 delivers growth factor mixture to the upstream section, at which point flow is possible only through an open pinch valve 210, 212, 214 or 216. The method of ECS operation occurs as follows. The programmable controller 701 activates the ECS pump 103 and pinch valve 210 simultaneously. Then growth factor mixture flows from the ECS feed reservoir 101, through the ECS pump 103, into line 104 and then into the upstream section of the first bioreactor 201. Because only pinch valve 210 is open, the only available flow path is through the first bioreactor 201. Product exits the first

bioreactors 201 when the ECS pump 103 is not activated, thus preventing transmembrane flow of media from the loop into the ECS product reservoir 102. After the first bioreactor 201 has completed its nutrient feed and product collection cycle, pinch valve 210 is deactivated and pinch valve 212 is immediately activated. During this transition, the ECS pump 103 continues to operate. The above described steps are repeated in a sequential fashion for the remaining bioreactors 201. After sequential nutrient feeding and product collection for each bioreactor 201, the ECS system remains at rest until the programmable controller 701 begins the series of operations again.

In order to accomplish the operation described above, the ECS pump 103 is peristaltic in operation, i.e., continuous contraction waves caused by the ECS pump 103 act on lines 104 and 105 to force contents onward. The peristaltic ECS pump 103 is arranged to have both lines 104 and 105 in the same ECS pump 103 at the same time so both lines 104 and 105 are acted on at the same time. Both lines 104 and 105 are of the same outside and inside diameter and consequently have the same flow rates. Finally, the ECS valves 210, 212, 214 and 216 are normally closed unless energized to open by a signal from the programmable controller 701.

The Loop Section 300

The loop section 300 is connected through: (1) lines 202 and 203 to the bioreactor section 200; (2) lines 304 and 305 to the loop feed waste section 400; and, (3) lines 306 and 307 to the oxygenator section 500. The loop section 300 includes two dissolved oxygen probes 308 and 309 with associated dissolved oxygen probe flow blocks 310 and 311, a loop heater 312, a loop flowmeter 313, a loop sampler 314, a loop

reservoir 315, a loop circulation pump 316, and a pH probe 332 and pH probe flow block 317.

The dissolved oxygen flow block 310 is connected by line 202 to the bioreactor section 200 and by line 318 to the loop heater 312 which is connected by the line 319 to the loop flowmeter 313. The flowmeter is connected by line 320 to the loop reservoir 315. The line 320 is fitted with a loop sampler 314.

Loop reservoir 315 is connected by line 321 to loop circulation pump 316. The loop section 300 is connected through lines 306 and 307 to the oxygenator section 500. A pH probe 332 and pH probe flow block 317 are located in line 322.

The dissolved oxygen probe flow block 311 is connected to the bioreactor section 200 by the line 203 and by the line 323 to the resistance temperature detector (RTD) probe flow block 324 having an associated RTD probe 325. The RTD probe flow block 324 is connected by the line 307 to the oxygenator section 500.

The Loop Feed/Waste Section 400

The loop feed/waste section 400 is connected by the lines 304 and 305 to the loop section 300. It includes a waste reservoir 401 connected to the loop reservoir 315 through the line 304; a basal medium feed reservoir 402 connected to the line 305 through the loop feed pump 403; and a glucose reservoir 404 connected by the line 406 through the glucose feed pump 405 to the line 305.

The Oxygenator Section 500

The oxygenator section 500 is connected by lines 306 and 307 to the loop section 300 and by the line 501 to the gases section 600. The oxygenator section 500 includes a hollow fiber oxygenator 502 having manifolds 504 and 505. The manifold 505 is connected

to th line 307 from the loop section 300. The line 306 is fitted with a loop filter 506 positioned before the manifold 504. Oxygenator exhaust line 507 is provided with an exhaust filter 508.

The Gases Section 600

The gases section 600 is connected to the oxygenator section 500 by the line 501. It includes a source of nitrogen, e.g., a cylinder 601, a source of carbon dioxide, e.g., a cylinder 602, and an air pump 603, each connected to manifold line 604 by lines 605, 606 and 607, respectively. Each of lines 605, 606 and 607 is fitted with an oil/particle gas filter 608 and a gas flowmeter 609.

Manifold line 604 is connected through line 610 to gas humidifier 611. The line 610 has an in-line gas feed filter 612. A humidifier water fill line 614 with an in-line filter 613 is attached to line 610.

The Electronics Section 700

The electronics section 700 includes a programmable controller 701, dissolved oxygen monitors 702, a temperature monitor and controller 703, a pH monitor 704, a loop circulation pump controller 705, and a basal medium feed pump controller 706. These electronic components are functionally interconnected with relevant sections of the system as shown by the broken lines in Figures 1 and 12.

The electronic elements, per se, are commercially available items. The component interconnections are made in a known manner and can readily be accomplished by the skilled person.

DETAILED DESCRIPTION OF CERTAIN SYSTEM ELEMENTS

Except as specifically described herein, the various elements of the system depicted by Figures 1, 11 and 12 are of conventional design.

1. The Hollow Fiber Bioreactor 201

Hollow fiber bioreactors useful in the invention may be of known design. See, e.g., United States patents 4,804,628; 3,821,087; and 4,391,912.

A preferred hollow fiber membrane and bioreactors including such hollow fiber membranes are described in co-pending application of Gebhard and Veeramallu, Serial No. 355,590, filed June 3, 1989. Permeable membranes useful in the bioreactors of this invention may be selected to accommodate the particular cell propagation process or method in which the bioreactor is to be used. Various types of semi-permeable membranes are known. Such semi-permeable membranes may be cellulosic or of synthetic origin. Polysulfone, polymethyl methacrylate and blends of isotactic and syndiotactic polymethyl methacrylate are appropriate. Semi-permeable hollow fibers of the kind described in U.S. Patent 3,896,061 incorporated herein by reference are appropriate.

A preferred form of hollow fiber bioreactor is a dialysis device sold by Toray Industries, Tokyo, Japan.

2. The Dissolved Oxygen Probe and Probe Flow Block

Figure 2 schematically illustrates a dissolved oxygen probe 308 and associated flow block 310. As shown, these components include the dissolved oxygen probe 308 and the flow cell body 310 which functions as a flow block. Medium from the bioreactor section 200 enters the flow cell body 310 through the line 202. Fluid exits through the line 318. The dissolved oxygen probe 308 is fitted to the body 310 by the O-ring 333. A teflon membrane 329 is fitted to the distal end of the probe 308. An observation window 330 fitted with an O-ring seal 326 is provided. The annular space 327 between the probe 308 and the body 310 is provided with an appropriate, e.g., .2μm, filter 328.

The dissolved oxygen probe and associated flow block permits on-line measurement of the dissolved oxygen content of the media. As shown in Figure 1, one such device 309, 311 is positioned to measure the DO1 of the media prior to entry into the bioreactor 201 and a second such device 308, 310 measures media DO2 after passage through the bioreactor(s). In use, an air bubble is introduced sterilely through the filter 328 to displace medium below the teflon membrane 329 of the dissolved oxygen (DO) probe 308. The loop flow channel is arranged such that flow continues through the flow cell, as shown in Figure 2, through lines 202, the flow cell and line 318. Because the medium flow is maintained during introduction of the air bubble, the pressure in the bubble is consistent with that of the medium flowing through the loop.

Upon completion of the calibration, the air bubble is removed through the filter 328 and the filter sealed closed. The observation window 330 is provided to permit monitoring of the entire calibration process and of the integrity of the DO probe membrane 329.

Commercially available DO probes may be utilized. A preferred galvanic dissolved oxygen probe is available from Phoenix Electrode, 6103 Glenmont, Houston, Texas 77081, as item 025NG-15-80. The DO probe is appropriately connected to the electronic DO monitor 702.

Aerobic Efficiency

Aerobic efficiency is determined by simultaneously measuring the GUR of the bioreactor(s) and the dissolved oxygen differential (ΔDO) across the bioreactor(s) and using the value GUR/ΔDO to assess the performance of the bioreactor(s) in terms of product formation; product formation and GUR/ΔDO

relationships having been established through historical performance. For these values to have significance, other system operating parameters must be taken into account such as loop circulation rate, basal medium type, temperature and Go. For example, using a murine hybridoma system as a model with a loop circulation rate of 350 ml/min, Dulbecco's Modified Eagles high glucose [4500 mg/l] medium as the basal medium, a temperature of 37°C, and a Go value in the range of 200 to 300 mg/l, GUR/ΔDO values of 3 to 6 (mg/hr)/% air saturation indicate an aerobically efficient bioreactor with acceptable product output while GUR/ΔDO values of 10 to 12 (mg/hr)/% air saturation indicate a bioreactor with significant oxygen mass transfer problems, low productivity and potential for system cancellation.

Real time verification of cell metabolism in the bioreactor(s) is provided by using the ΔDO value as an indication of the oxygen consumed in the process of respiration. This method of metabolism verification is of exceptional value when a continuity of ΔDO has been established. Deviations to the established ΔDO continuity enable the system operator to immediately take appropriate corrective action without reconducting time consuming off line assays.

3. The Hollow Fiber Temperature Control System

Figure 3 is a schematic depiction of a preferred hollow fiber temperature control system useful in the invention.

As shown in schematic Figure 3, a hollow fiber bioreactor 201 is connected by the line A to the loop heater 312 which is connected by broken line B to the electronic temperature monitor and controller 703. A thermostat 331 is fitted b twe n the heater 312 and the line B.

The heat r 312 is connect d by the line C to the loop reservoir 315 which is connected by line D to the resistance temperature detector (RTD) probe block 324 and associated RTD probe 325. The RTD probe 325 is connected by the broken line E to the temperature monitor and controller 703. Line F connects the RTD probe flow block, through the loop circulation pump 316, to the oxygenator 502 which is connected by line G to the bioreactor(s) 201.

As Figure 3 shows, loop temperature is monitored by an RTD probe 325 inserted into the loop. The RTD probe 325 is coupled to the electronic monitor and controller 703. The controller 703 compares the signal from the RTD probe 325 to a preselected set point and makes adjustments accordingly to a heater 312, preferably mounted externally to a stainless steel portion of the loop.

4. The Basal Medium and Reagent Control System

The basal medium and reagent control system is schematically shown by Figure 4. Glucose and ammonia analyzer 801 and lactate analyzer 813 are coupled by line H to the basal medium feed pump controller 706 and by line I to the programmable controller 701. The basal medium feed pump controller 706 is coupled by broken line L to the basal medium feed pump 403. The programmable controller 701 is coupled by broken line J to the glucose feed pump 405 and by broken line K to the ECS pump 103.

The glucose and ammonia analyzer 801 is an off line analyzer with multiple dry film based chemistry functions. Samples taken from the loop sample port 314 are manually transferred to the analyzer 801 for glucose and ammonia analysis. Glucose and ammonia assays must be performed independently on the form of analyzer shown.

The off line lactat analyzer 813 c nsists of a spectrophotom ter and analytical means designed specifically for the quantification of lactate through spectrophotometric analysis. Samples taken from the loop sample port 314 are manually transferred to the lactate analyzer 813 for lactate analysis.

Data provided by line H from the analyzers 801 and 813 to the basal feed medium pump controller provides information useful to achieve either manually as shown, or by automation, introduction of basal medium into the loop at a rate required for a preselected GUR. Preselection of the GUR is based on historical data obtained with a similar hollow fiber system. Data provided by the line I to the programmable controller 701 achieves introduction of glucose into the loop at a rate required to maintain a specific Go and preselected GUR.

5. The Waste Disposal System

A waste disposal system useful in the invention is schematically shown in Figure 5.

As shown in Figure 5, the bioreactor 201 is part of a loop which originates and terminates in the loop reservoir 315. Medium feed line M is connected to the loop line N. Loop pick-up line N extends through the loop circulation pump 316 to the hollow fiber oxygenator 502. Line O connects the oxygenator 502 to the hollow fiber reactor 201. As shown, the line L extends into the medium reservoir 315 and terminates at a point above the level of the medium 807 contained therein.

Drain line 803 extends from near the bottom of reservoir 315, through the drain line clamp 804 to a connection with the waste line 806 which extends from the waste exist port 808 at the top of the reservoir 315 and which is provided with waste line clamp 805.

A normally closed .2μm filter vent 802 is located on the reservoir above the drain line 803.

A waste displacement system is thus provided, whereby volumes of fresh basal medium entering the loop through line M automatically displace substantially identical volumes of spent medium in the loop through a waste exit port 808 and into waste line 806. Clamp 805 is open during normal operation. Siphoning, caused by venting across the oxygenator membranes, is prevented by the level of medium 807 falling below waste exit port 808 and causing a siphon break.

Reservoir drain line 803 is provided to allow for draining of the reservoir medium. To drain the reservoir 315, the normally closed drain line clamp 804 is opened and the normally open waste line clamp 805 is closed and the normally closed .2μm filter vent 802 is opened. The reservoir can then be drained by siphon.

6. The Oxygenator

Figure 6 schematically illustrates the hollow fiber oxygenator. As shown in Figure 6, oxygen depleted medium travels through loop line 306 which is coupled to oxygenator manifold 504. Medium then enters a plurality of hollow fibers 503 formed, e.g., from polyethylene or polypropylene held in place by potting material 510. Gases, usually air, nitrogen and carbon dioxide, are blended, humidified, particulate and sterile filtered prior to entering the ECS 509 of the oxygenator through line 501. Gas exchange occurs across the hollow fiber membranes causing the dissolved gases in the medium to come into equilibrium with the gas mixture introduced into the ECS 509. Oxygen replenished medium exits the hollow fibers 503 and then collects in manifold 505 and is delivered to loop line 307. Depleted ECS

gases exit the oxygenator ECS 509 through oxygenator exhaust line 507. The air functions primarily as an oxygen source, nitrog n is useful to reduce oxygen levels and thus ameliorate oxygen toxicity problems and carbon dioxide functions as a pH control agent.

7. The Loop Heater

A preferred form of the loop heater 312 is shown in cross-section by Figure 7. As illustrated, the heater comprises a stainless steel tube 809, an etched foil resistance heating element 810 and appropriate insulation 811. The heater is provided with suitable wiring 812 and a thermostat 331 as a safety feature. The medium 813 passes through the tube 809 in a flow direction indicated by arrows.

8. Bioreactor System

A preferred form of compact bioreactor system configuration is shown by Figure 8. In the figure, elements shown in broken line are positioned behind elements shown in solid lines. All elements are directly or indirectly mounted on a transparent polycarbonate or similar sheet.

THE METHOD OF THE INVENTION

The method of the invention is described in general by reference to the figures.

Typical ECS feed material is placed in the ECS feed reservoir 101. As heretofore described, such material may comprise basal medium, growth factors and/or serum.

Typical basal feed material as heretofore described is placed in the basal feed res_rvoir 402. Such material may comprise basal medium, L-glutamine and antibiotics.

A glucose solution, preferably an aqueous solution contained from about 150 g/l to about 300 g/l glucose, is placed in glucose res rvoir 404.

A cell line such as murine hybridoma cell line is propagated in the system as follows: The system excluding the oxygenator 502 and bioreactor 201, which can include multiple bioreactors, as shown in Figures 11 and 12, is sterilized by autoclaving. Presterilized oxygenator 502 and bioreactor(s) 201 are incorporated into the loop under a laminar flow hood. Following assembly, the system is placed in a chamber designed specifically to provide system support functions. Electronic cables (broken lines) are provided which connect the loop circulation pump 316, pH probe 332, dissolved oxygen probes 308 and 309, RTD probe 325 and loop heater 312 to the I/O panel of the control chamber. ECS pump 103, basal medium feed pump 403, and glucose feed pump 405 are provided as functional components of the chamber. The oxygenator gas feed line 501 is connected to a chamber port which provides the appropriate premixed gases. Basal medium feed reservoir 402, waste reservoir 401, glucose reservoir 404, ECS feed reservoir 101 and ECS product reservoir 102 having had their contents adjusted appropriately are connected to the system now in place in the chamber.

Once the aforementioned connections have been made the loop section 300 is filled by clamping the loop line 321 downstream of the loop reservoir and activating the basal medium feed pump 403. This action causes the loop to fill in one specific direction and results in a priming of the loop circulation pump 316. The clamp is opened after the loop is completely filled and the loop reservoir 315 is one third filled. At this point the loop circulation pump 316 is activated and the loop circulation flow rate typically set at 350 ml/min per bioreactor for bioreactor(s) with 2.0 m^2 surface area. Concurrent with this activity the ECS of the

bioreactor(s) 201 is filled by manually overriding the ECS control program at programmable controller 701. The ECS pump 103 is activated to pump ECS medium into the bioreactor(s) thereby displacing air from the ECS into the ECS product reservoir 102. This action is continued until the ECS is completely filled with medium. After filling the ECS, the ECS control program is reinstated. The rate of ECS feed and product removal is dependent upon bioreactor surface area, product stability, specific nutrient requirements, and targeted GUR. Maintenance of Go at about one millimolar is appropriate. Simultaneous introduction of growth factor mixture into and removal of product from the ECS at rates that do not differ by more than about five percent is achieved by the programmable controller 701 directing the ECS pump 103. A typical rate of ECS feed and product removal for a murine hybridoma in this system format is 60 ml/day per bioreactor. The ECS product reservoir is collected and replaced as required.

Dissolved oxygen probes 308 and 309 are calibrated by the method described under Detailed Description of Certain System Elements. The DO probes 308 and 309 are calibrated biweekly as part of regular system maintenance. A sample of loop medium is taken from the loop sampler 314 and used to make an off line temperature determination. The temperature controller 703 is adjusted to the off line measured value. At this point the loop heater 312 function is activated by setting the heat control set point. A set point is selected which will maintain a constant system loop temperature of 37°C. Another sample of loop medium is taken from the loop sampler 314 and used to make an off line pH measure- m nt. The pH monitor 704 is then adjust d to be consistent with the off line measured value.

After the aforementioned process parameters have been established the system is allowed a 48 hour check period during which time the probes are allowed to stabilize, system function is verified, and sterility is verified. The basal medium feed pump controller 706 is adjusted to deliver approximately 3 liters of basal medium to the system over the 48 hour check period to serve as a system flush and to remove packing fluids from the bioreactor(s). Upon completion of the 48 hour check period, an inoculum of hybridomas is prepared. The inoculum generally consists of approximately 2.25×10^8 cells/m^2 surface area which are suspended in a growth factor mixture which is in turn placed in the ECS feed reservoir 101. The ECS control program is manually overridden to allow for transfer of the inoculum from the ECS feed reservoir 101 through line 104 to the ECS of the bioreactor(s) 201. After completion of the inoculation process, a new ECS feed reservoir 101 containing growth factor mixture is exchanged for the empty inoculation reservoir. A new ECS product reservoir 102 is put in place at this point. Immediately following inoculation, the rate of basal medium delivery to the loop is set by adjusting the basal medium feed pump controller 706 to a value selected on the basis of bioreactor surface area, basal medium type, cell line requirements and targeted GUR. A typical basal medium feed rate for a murine hybridoma with a targeted plateau GUR of 150 mg/hr is 42 ml/hr in bioreactor(s) with 2.0 m^2 surface area available using Dulbecco's Modified Eagles high glucose medium. Glucose addition as a function separate from basal medium is optional in this system format and requir s programming the programmable controller 701 to affect the correct delivery of glucose from the glucose feed reservoir 404 to the loop via the glucose feed pump 405.

Typical concentrations of gluc s in the glucose feed reservoir 404 range between 150 g/l to 300 g/l. Separation of glucose addition allows for greater control of Go and subsequently greater process control. When glucose is contained in the basal medium, Go values typically settle in a range of 200 mg/l to 300 mg/l. However, when glucose addition is separated from basal medium feed, Go values can be independently controlled at as low as 50 mg/l. Go samples are taken every 24-48 hours from the loop sampler port 314 for off line analysis. Significant plateauing of GUR and ΔDO values occur approximately 10 days after inoculation. At this point basal medium feed rate and/or glucose feed rate may undergo fine adjustments to help stabilize GURs. See Figure 9.

After plateauing, GUR in the predescribed format can remain constant without further system adjustments for 65-85 days with diligent system maintenance. ΔDO values serve as a real time verification of system performance by providing an instantaneous assessment of the metabolic state of the cell mass without performing off line assays and provide a method to instantaneously assess protein production. ΔDO values are used to monitor the onset of performance problems associated with mass transfer limitations which affect aerobic efficiency. The time of onset of these problems is dependent upon cell type, bioreactor size, and plateau GUR values. The system parameters set forth here would normally incur significant performance loss due to mass transfer limitations at approximately day 70. Aerobic efficiency of the system is determined by the value GUR/ΔDO where productive plateau values fall in a range of 3-6(mg/hr)/% air saturation and system cancellation is affected when this value reaches 10-12 (mg/hr)/% air saturation. See Figure 10.

EXEMPLIFICATION OF THE INVENTION

Example I

The propagation of an anchorage dependent type cell line in a single bioreactor hollow fiber cell propagation system of the invention is described in this example. The cell line is a Crandall feline kidney (CRFK) Dixon strain fibroblast that persistently sheds feline immunodeficiency virus (FIV) and associated proteins. The product of interest in the culture supernatant is a viral core glycoprotein p28, quantified by enzyme linked immunoadsorbent assay (ELISA).

The culture system was sterilized, assembled, connected to accessories and chamber controls, primed and flushed, as generally described in "The Method of the Invention" section.

The process parameters in the culture system were set to establish start up conditions. The set point for the temperature controller 703 was set at 37°C. The gas flow meters 609 were adjusted such that the pH monitor 704 read 7.25, and the DO monitors 702 read about 70% air saturation. The loop circulation controller 705 was set to obtain a loop circulation rate of 400 ml/min. The programmable controller 701 was set to deliver growth factor mixture (and collect ECS product) at a frequency of 6 times/day and a volume of 50 ml/day.

An inoculum containing $4.5X10^8$ cells was pumped into the ECS of the bioreactor 201 as generally described in "The Method of the Invention" section. A $2.0m^2$ surface area bioreactor (model FC-20, Toray Industries) 201 was used in this run. The growth factor mixture comprised 40% fetal bovine serum, 2% L-glutamine, 1% penicillin streptomycin, in Dulbecco's Modified Eagle's (DME) high glucose (4500 mg/l) basal medium. This composition was maintained

throughout the run. The ECS fe d reservoir 101 and ECS product reservoir 102 were replaced three times/week throughout th run.

The basal medium feed pump controller 706 was set to deliver 41 ml/hr. The basal feed medium comprised 2% L-glutamine, 1% penicillin streptomycin, in DME high glucose basal medium. This composition was maintained throughout the run. Thus, the optional separate glucose feed system was not used in this run. The basal medium feed reservoir 402 was replaced 3 times/week, and the waste reservoir 401 was replaced when necessary.

Samples were withdrawn regularly for off line glucose analysis, as generally described in "The Method of the Invention" section. As Table 1 shows, the glucose utilization rate (GUR) and the dissolved oxygen differential across the bioreactor (ΔDO) both increased as the cells proliferated in the ECS of the bioreactor 201.

TABLE 1

Day #	GUR (mg/hr)	ΔDO (%)	p28 (μg/day)
1	10.000		
2	15.000		
3	15.600		
4			
5			
6	32.700		
7	28.500	0.300	
8	41.700	0.600	150.000
9	44.200	1.500	
10	48.100	2.200	100.000
11			
12			
13	51.280		350.000
14	50.600		
15	47.200	9.900	350.000

Day #	GUR (mg/hr)	ΔDO (%)	p28 (μg/day)
16	47.200	13.500	
17	52.400	14.100	375.000
18			
19		16.000	
20	59.500	15.800	500.000
21	66.000	14.800	
22	61.500	13.800	300.000
23	63.500	14.400	
24	67.300	14.200	300.000
25	67.900	15.600	
26			
27		23.800	
28	71.800	21.100	360.000
29	60.300	22.400	
30	51.900	22.400	
31	55.100	21.400	292.000

The target GUR was set at 60 mg/h. By day 13 the GUR and p28 protein production stabilized significantly. The production phase that followed was maintained for almost three weeks. The GUR, p28 production and ΔDO profiles were all relatively stable and parallel during this period.

The goals of this run were to demonstrate an ability to culture this anchorage dependent cell line in the invention, and to manufacture enough viral p28 protein to meet in house research requirements. Having met these objectives, the run was terminated.

Example II

The propagation of a suspension type cell line in a single bioreactor hollow fiber cell propagation system of the invention is described in this example. The cell line is a murine hybridoma that secretes monoclonal antibodies against transmissible gastro enteritis virus (TGEV). The IgG_{2a} antibody product in the culture supernatant is measured by radial immuno diffusion (RID) assay.

The culture system was sterilized, assembled, connected to accessori s and chamber controls, primed and flushed, as generally described in "The Method of the Invention" section.

The process parameters in the culture system were set to establish start up conditions. The set point for the temperature controller 703 was set at 37°C. The gas flow meters 609 were adjusted such that the pH monitor 704 read 7.31, and the DO monitors 702 read about 95% air saturation. Nitrogen flowrate in this run was set to zero. The loop circulation controller 705 was set to obtain a loop circulation rate of 350 ml/min. The programmable controller 701 was set to deliver growth factor mixture (and collect ECS product) at a frequency of 6 times/day and a volume of 24 ml/day.

An inoculum containing $3.1X10^8$ cells was pumped into the ECS of the bioreactor 201 as generally described in "The Method of the Invention" section. A $2.0m^2$ surface area bioreactor (model FC-20, Toray Industries) 201 was used in this run. The growth factor mixture comprised 35% fetal bovine serum, 1% penicillin streptomycin, in DME high glucose basal medium. This composition was maintained throughout the run. The ECS feed reservoir 101 and ECS product reservoir 102 were replaced three times/week throughout the run.

The basal medium feed pump controller 706 was set to deliver 50.4 ml/hr. The basal feed medium comprised 2% L-glutamine, 1% penicillin streptomycin, in DME high glucose basal medium. This composition was maintained throughout the run. Thus, the optional separate glucose feed system was not used in this run. The basal medium feed res rvoir 402 was replaced 3 times/week, and th waste r servoir 401 was replaced when necessary.

Samples wer withdrawn regularly for off line glucose analysis, as generally described in "The Method of the Invention" section. As Table 2 shows, the GUR and ΔDO both increased as the cells proliferated in the ECS of the bioreactor 201.

TABLE 2

Day #	GUR (mg/hr)	ΔDO (%)	Ab (mg/day)
1	9.570		
2			
3	37.200		1.920
4	50.900		
5	66.000		8.640
6	94.460		
7	110.750	14.000	32.160
8			
9			
10	155.700	11.600	47.520
11	148.800	15.200	
12	161.200	13.300	64.320
13	149.800	13.200	
14	145.500	12.100	90.480
15			
16			
17	137.500	11.300	
18	143.900		124.560
19			
20	168.600		
21	168.000	26.400	135.120
22			
23			
24	115.600	24.700	
25	127.450	33.500	135.120
26	142.200	34.100	
27	153.180	33.100	
28	156.900	27.200	140.400
29			
30			
31	155.290	38.600	151.440
32	145.600	34.300	
33	145.600	34.300	156.960
34	146.000	28.500	
35	146.850	30.900	162.720
36			
37			
38	147.700	32.800	168.480
39	147.200		
40	148.100	34.100	204.936

Day #	GUR (mg/hr)	ΔDO (%)	Ab (mg/day)
41	149.380	31.900	
42	149.380	29.400	189.600
43			
44			
45	151.500	32.000	194.664
46	151.500	27.100	
47	151.500	35.400	179.736
48	153.100	32.100	
49	153.300	29.400	179.736

The basal medium feed rate was manipulated to establish and maintain a target GUR of 150 mg/hr. By day 10 the GUR stabilized significantly, and by day 22, the antibody production [Ab} and ΔDO stabilized too. The production phase following immediately was maintained for four weeks, during which, the antibody production to ΔDO ratio exhibited relative constancy.

The goals of this run were to demonstrate an ability to culture this suspension type cell in the invention, and to manufacture enough antibody to meet in house research requirements. Having met these objectives, the run was terminated.

Example III

The propagation of a suspension type cell line in a single bioreactor hollow fiber cell propagation system of the invention is described in this example to illustrate improved productivity by means of controlling the effects of product and metabolite feedback inhibition. The cell line is a murine hybridoma that secretes monoclonal antibodies against TGEV. The IgG_{2a} antibody product in the culture supernatant is measured by RID assay.

The culture system was sterilized, assembled, connected to accessories and chamber controls, primed and flushed, as generally described in "The Method of the Invention" section.

The process parameters in the culture system were set to establish start up conditions. The set point for the temperature controller 703 was set at 37°C. The gas flow meters 609 were adjusted such that the pH monitor 704 read 7.3. Nitrogen flowrate in this run was set to zero. DO monitors 702 were excluded in this run. The loop circulation controller 705 was set to obtain a loop circulation rate of 350 ml/min. The programmable controller 701 was set to deliver growth factor mixture (and collect ECS product) at a frequency of 6 times/day and a volume of 24 ml/day. This volumetric feed rate was varied through the run as described below.

An inoculum containing $3X10^8$ cells was pumped into the ECS of the bioreactor as generally described in "The Method of the Invention" section. A $1.4m^2$ surface area bioreactor (model 1-L, C.D. Medical Inc.) 201 was used in this run. The growth factor mixture comprised 35% fetal bovine serum, 1% penicillin streptomycin, in DME high glucose basal medium. This composition was varied through the run as described below. The ECS feed reservoir 101 and ECS product reservoir 102 were replaced three times/week throughout the run.

The basal medium feed pump controller 706 was set to deliver 41.5 ml/hr. The basal feed medium comprised 2% L-glutamine, 1% penicillin streptomycin, in DME high glucose basal medium. This composition was maintained throughout the run. Thus, the optional separate glucose feed system was not used in this run. The basal medium feed reservoir 402 was replaced 3 times/week, and the waste reservoir 401 was replaced when necessary.

Samples were withdrawn regularly for off line glucose analysis, as generally described in "The Method of the Invention" section. As Table 3(a) shows, the GUR and ΔDO both increased as the cells proliferated in the ECS of the bioreactor 201.

TABLE 3(a)

Day #	GUR (mg/hr)	ECS (ml/day)	Ab (mg/day)
1	12.0	24	
2	31.9	24	
3		24	
4	45.2	24	17.28
5	52.7	24	
6	53.5	24	50.40
7	83.8	24	
8	100.4	24	43.68
9	91.7	24	
10	100.0	24	
11	103.8	24	80.16
12	84.2	24	
13	88.5	24	88.32
14	82.9	24	
15	80.8	24	88.32
16		24	
17		24	
18	81.2	24	88.32
19	85.7	24	
20	100.1	24	88.32
21	105.2	24	
22	109.7	24	96.48
23		24	
24	105.8	24	
25		24	
26	104.0	24	121.44
27	102.1	30	
28	103.4	30	
29	103.1	30	151.80
30		30	
31		30	
32	109.1	30	151.80
33	108.6	30	
34		30	130.80
35	110.7	42	
36	112.8	42	168.84
37		42	
38		42	

Day #	GUR (mg/hr)	ECS (ml/day)	Ab (mg/day)
39	114.6	42	168.84
40	114.6	42	
41	114.6	42	154.56
42		42	
43	113.1	42	153.72
44		54	
45		54	
46	114.3	54	163.08
47	113.7	54	
48	113.7	54	163.08
49	114.6	54	
50	114.6	54	163.08
51		60	
52		60	
53	114.9	60	162.00
54	115.5	60	
55	115.5	60	162.00

The basal medium feed rate was manipulated to establish and maintain a target GUR of 100 mg/hr. By day 8 the GUR stabilized significantly, and by day 26, the antibody production stabilized too.

The production phase following immediately was maintained for almost four weeks. During this period, the ECS volumetric feed rate (and ECS product collection rate) was maintained at 30,42 and 54 ml/day, each for at least a week. The fetal bovine serum content of the growth factor mixture was adjusted to ensure constant serum feed to the system. As Table 3(b) shows, while the increase in ECS feed rate had no effect upon GUR, it improved the antibody productivity by 45%.

TABLE 3(b)

ECS (ml/day)	Ab (mg/day)
OLDER RUN	
24.000	112.800
24.000	96.000
24.000	108.000
36.000	162.000
36.000	154.800

ECS (ml/day)	Ab (mg/day)
36.000	144.000
36.000	144.000
36.000	144.000
48.000	139.200
CURRENT RUN	
24.000	96.480
24.000	121.440
30.000	151.800
30.000	151.800
30.000	130.800
42.000	168.840
42.000	168.840
42.000	154.560
42.000	153.720
54.000	163.080
54.000	163.080
54.000	163.080
60.000	162.000
60.000	162.000

Thus, the effects of product and metabolite feedback inhibition were overcome by maintaining a higher ECS product collection rate (or ECS feed rate). The combined results of this exercise and that from another run are reported in Table 3(b). It was concluded that for the given set of process conditions, the ECS feed rate must be set to at least 42 ml/day to maximize antibody productivity.

The goal of this run was to illustrate improved productivity by means of controlling the effects of product and metabolite feedback inhibition. Having met this objective, the run was terminated.

Example IV

The propagation of a suspension type cell line in a single bioreactor hollow fiber cell propagation system of the invention is described in this example to illustrate improved process control by means of an

optional separate glucose feed system. The cell line is a murine hybridoma that secretes monoclonal antibodies against TGEV. The IgG_{2a} antibody product in the culture supernatant is measured by RID assay.

The culture system was sterilized, assembled, connected to accessories and chamber controls, primed and flushed, as generally described in "The Method of the Invention" section.

The process parameters in the culture system were set to establish start up conditions. The set point for the temperature controller 703 was set at 37°C. The gas flow meters 609 were adjusted such that the pH monitor 704 read 7.2, and the DO monitors 702 read about 95% air saturation. Nitrogen flowrate in this run was set to zero. The loop circulation controller 705 was set to obtain a loop circulation rate of 350 ml/min. The programmable controller 701 was set to deliver growth factor mixture (and collect ECS product) at a frequency of 6 times/day and a volume of 35 ml/day.

An inoculum containing $3X10^8$ cells was pumped into the ECS of the bioreactor 201 as generally described in "The Method of the Invention" section. A 2.0 m^2 surface area bioreactor (model FC-20, Toray Industries) was used in this run. The growth factor mixture comprised 24% fetal bovine serum, 1% penicillin streptomycin, in DME glucose free basal medium. This ECS feed composition was maintained throughout the run. The ECS feed reservoir 101 and ECS product reservoir 102 were replaced three times/week throughout the run.

The basal medium feed pump controller 706 was set to deliver 42 ml/hr. The basal feed medium comprised 2% L-glutamine, 1% penicillin str ptomycin, in DME

glucose free basal m dium. This composition was maintained throughout the run. The programmable controller 701 was set to deliver glucose, via the glucose feed pump 405, such that the effective glucose concentration in the feed medium (Gi) was 1198 mg/l. The glucose reservoir 404 contained glucose stock solution at 200 g/l. The basal medium feed reservoir 402 was replaced 3 times/week, the glucose reservoir was replaced 1 time/week, and the waste reservoir 401 was replaced when necessary.

Samples were withdrawn regularly for off line glucose analysis, as generally described in "The Method of the Invention" section. As shown in Table 4 below, the Gi was manipulated via the programmable controller 701, to support the increasing GUR and ΔDO of the proliferating cells in the bioreactor 201, and at the same time maintain an approximately constant system glucose concentration (Go) of 800 mg/l.

TABLE 4

Day #	Gi (mg/1)	Go (mg/1)	GUR (mg/hr)	ΔDO (%)	Ab (md/day)
1	1198	790.0	17.1		
2	2001	770.0	51.6	6.2	
3	2489	840.0	69.3	9.6	
4	2716	840.0	79.0	11.2	
5	2845	830.0	84.8	12.1	
6	2923	830.0	88.1	13.7	
7	2975	780.0	92.4	16.1	40.950
8	3148	840.0	97.4	18.0	
9	2606	360.0	94.3	18.6	62.300
10	2489	270.0	93.2	21.0	
11					
12	2489	240.0	94.5	22.6	82.950
13	2489	210.0	95.7	25.1	
14	2489	157.0	97.9	29.6	93.100
15	2489	123.0	99.4	33.1	

Day #	Gi (mg/l)	Go (mg/l)	GUR (mg/hr)	ΔDO (%)	Ab (md/day)
16	2489	107.0	100.0	33.5	115.150
17					
18	2489	86.0	100.9	35.1	
19	2489	86.0	100.9	35.6	127.050
20	2489	80.0	101.2	37.1	
21	2489	68.0	101.7	38.1	133.000
22	2489	68.0	101.7	38.6	
23	2489	51.0	102.4	40.6	133.000
24					
25					
26	2489	49.0	102.5	41.0	144.900
27	2489	53.0	102.3	43.0	
28	2489	48.0	102.5	44.1	148.750
29	2489	50.0	102.4		
30	2489	48.0	102.5	41.6	154.000
31					
32					
33	2489	47.0	102.5	44.6	154.000
34	2489	51.0	102.4	44.7	
35	2489	49.0	102.5	45.1	148.750

By day 8 the GUR reached a target value of 100 mg/hr, at which point, the glucose pump was momentarily shut off to rapidly bring Go down to 300 mg/l. Then, the programmable controller 701 was reset to obtain a Gi of 2489 mg/l, to support the established GUR of 100 mg/hr, and at the same time allow the Go to settle below 1 mM (or 180 mg/l).

The basal medium feed pump controller 706 was set to deliver 42 ml/hr from the beginning of the run, a basal medium feed rate that can support a GUR of 150 mg/hr. However, immediately following the Go manipulation on day 8, the GUR levelled at 100 mg/hr, and ΔDO at 22%, as a result of glucose limitation (200 mg/l <Go<300 mg/l). Thus, a separate glucose feed system made possible an independent and useful control of the process. After day 14, further glucose limitation (Go<180 mg/l) led to an increasingly aerobic GUR as a result of the Pasteur shift in cell metabolism. Thus, over the next three weeks, although

the GUR remained relatively unchanged at 100 mg/hr, the ΔDO eventually stabilized at 44%. The antibody production stabilized at 154 mg/hr, which is comparable to production values obtained under similar process conditions but non limiting glucose values (Go>300 mg/l), as seen in Example 3.

The goal of this run was to demonstrate an improved ability to control the process by means of using a separate glucose feed system. Having met this objective, the run was terminated at this point.

EXAMPLE V

The propagation of an anchorage dependent cell line in both single and multiple bioreactor hollow fiber cell propagation systems of the invention is described in this example to illustrate scale-up of protein production. A single bioreactor system and a four bioreactor system were each run using a recombinant chinese hamster ovary (rCHO) cell line. The product of interest in the culture supernatant is a 70-90 Kd proteoglycans, a glycoprotein, quantified by ELISA.

Single Reactor Run

A single 2.0 m^2 surface area bioreactor (model FC-20, Toray Industries) 201 was used for this run. The culture system was sterilized, assembled, connected to accessories and chamber controls, primed and flushed, as described in "The Method of the Invention" section. The process parameters in the culture system were set to establish start up conditions. The set point for the temperature controller 703 was set to 37°C. The gas flowmeters 609 were adjusted such that the pH monitor 704 read 7.26, and the DO monitors read about 65% air saturation. The loop circulation controller 705 was set to obtain a loop circulation rate of 400 ml/min.

The programmable controller 701 was set to deliver growth factor mixture (and collect ECS product) at a frequency of 6 times/day and a volume of 50 ml/day.

An inoculum containing $4.5x10^8$ cells was pumped into the ECS of the bioreactor 201 as described in "The Method of the Invention" section. The growth factor mixture comprised 40% fetal bovine serum, 1% penicillin streptomycin, in Dulbecco's Modified Eagle's (DME) high glucose (4500 mg/l) basal medium. The basal feed medium comprised 2% L-glutamine, 1% penicillin streptomycin, 1% non-essential amino acids (NEAA) in DME high glucose basal medium. The basal medium feed pump controller 706 was set to deliver 40 ml/hr. Production rate of proteoglycans was relatively stable for seven weeks at an average of 141 μg/day.

Four Reactor Run

Scale-up was accomplished by using four 2.0 m^2 surface area bioreactors (model FC-20, Toray Industries) 201 in a multiple bioreactor configuration as described in "The System of the Invention" section. The culture system was sterilized, assembled, connected to accessories and chamber controls, primed and flushed, as described in "The Method of the Invention" section.

The temperature, pH and DO were set to duplicate conditions in the single bioreactor run. The loop circulation controller 705 was set to obtain a loop circulation rate of 1400 ml/min (350 ml/min per bioreactor). The programmable controller 701 was set to deliver bioreactor ECS feed medium (and collect ECS product) at a frequency of 24 times/day and a volume of 256 ml/day (64 ml/day per bioreactor).

An inoculum containing 1.8x 10^9 (4.5x10^8 per bioreactor) cells was pump d into the ECS of the bioreactors 201 as described in "The Method of the Invention" section. The growth factor mixture comprised 40% fetal bovine serum, 1% penicillin streptomycin in Dulbecco's Modified Eagle's (DME) high glucose (4500 mg/l) basal medium. The basal feed medium comprised 2% L-glutamine, 1% penicillin streptomycin, 1% NEAA in DME high glucose basal medium. The basal medium feed pump controller 706 was set to deliver 166.7 ml/hr (41.6 ml/hr per bioreactor). Production rate of proteoglycans averaged 870 μg/day over a three week period.

The scale-up factor for protein production was calculated at 6.2. On a per bioreactor basis, an ECS feed rate (and product collection rate) of 64 ml/day was used for the four bioreactor run as opposed to 40 ml/day in the single bioreactor run. The concentrations of proteoglycans in both runs were comparable.

EXAMPLE VI

The propagation of an anchorage dependent cell line in a multiple bioreactor hollow fiber cell propagation system of the invention is described in this example to illustrate scale-up of protein production. Scale-up was accomplished by using four 2.0 m^2 surface area bioreactors (model FC-20, Toray Industries) 201 in a multiple bioreactor configuration as described in "The System of the Invention" section. The cell line is a CRFK Dixon strain fibroblast that persistently sheds FIV and associated proteins, as described in Example I above. The product of interest in the culture supernatant is a viral core glycoprotein p28, quantified by ELISA.

The cultur system was sterilized, assembled, connected to accessories and chamber controls, primed and flushed, as described in "The Method of the Invention" section. The temperature, pH and DO were set to duplicate conditions as used for Example I. The loop circulation controller 705 was set to obtain a loop circulation rate of 1400 ml/min (350 ml/min per bioreactor). The programmable controller 701 was set to deliver growth factor mixture (and collect ECS product) at a frequency of 6 times/day and a volume of 200 ml/day (50 ml/day per bioreactor).

An inoculum containing 1.09×10^9 (2.6×10^8 per bioreactor) cells was pumped into the ECS of the bioreactors 201 as described in "The Method of the Invention" section. The growth factor mixture comprised 25% fetal bovine serum, 2% L-glutamine, 1% penicillin streptomycin, in Dulbecco's Modified Eagle's (DME) high glucose (4500 mg/l) basal medium. The basal feed medium comprised 2% L-glutamine, 1% penicillin streptomycin, in DME high glucose basal medium. The basal medium feed pump controller 706 was manipulated and eventually set to deliver 166 ml/hr (41.5 ml/hr bioreactor). This basal medium feed rate corresponds to a scale-up factor of 4 over that used in the single bioreactor system in Example 1. Production rates of p28 protein were relatively stable for six weeks, and reached up to 1208 μg/day. An average production rate of 366 μg/day was seen during the production phase in Example 1. Thus, the production scale-up factor for p28 protein was 3.3.

EXAMPLE VII

The propagation of a suspension type cell line in a multiple bioreactor hollow fiber cell propagation system of the invention is described in this example. The express purpose of this run was to test the

ability of the lumenal and ECS manifold system to deliver nutri nts evenly to each of the bioreactors in a multiple bioreactor configuration, rather than a demonstration of scale-up of protein production. One system having two banks of four bioreactors was used, each bank containing four 2.0 m^2 surface area bioreactors (model FC-20, Toray Industries) 201 in a configuration as described in "The System of the Invention" section. The cell line was a murine hybridoma that secretes monoclonal antibodies against TGEV, as described in Example II above. The IgG_{2a} antibody product in the culture supernatant is measured by RID assay.

The culture system was sterilized, assembled, connected to accessories and chamber controls, primed and flushed, as described in "The Method of the Invention" section. The temperature, pH and DO were set to duplicate conditions in Example II. The loop circulation controller 705 was set to obtain a loop circulation rate of 2000 ml/min (250 ml/min per bioreactor). The programmable controller 701 was set to deliver growth factor mixture (and collect ECS product) at a frequency of 6 times/day and a volume of 288 ml/day (36 ml/day per bioreactor).

An inoculum containing $3.2x10^9$ ($4.0x10^8$ per bioreactor) cells was pumped into the ECS of the bioreactors 201 as described in "The Method of the Invention" section. The growth factor mixture comprised 40% fetal bovine serum, 2% L-glutamine, 1% penicillin streptomycin, in Dulbecco's Modified Eagle's (DME) high glucose (4500 mg/l) basal medium. The basal feed medium comprised 2% L-glutamine, 1% penicillin streptomycin, in DME high glucose basal medium. The basal medium feed pump controller 706 was set to deliver 333 ml/hr (41.6 ml/hr per bioreactor).

On day 15, the ECS of each bioreactor was sampled individually and assayed for antibody concentration. In the first multiple bioreactor set, the antibody concentration was 2.33 ± 0.21 mg/ml. In the second multiple bioreactor set, the antibody concentration was 1.83 ± 0.21 mg/ml. Thus, the percentage variation in antibody concentration for the first and second set is 6.55% and 11.24%, respectively. This experimental result essentially represents an identical product concentration in different bioreactors in the set. The premise is that in each multiple bioreactor set, an equal concentration of product in each bioreactor means that there must be even cell growth in each bioreactor, which is possible only if there is an even distribution of nutrients to each bioreactor 201. Thus, the functional efficacy of the lumenal and ECS manifold system was demonstrated.

Claims:

1. A hollow fiber cell propagation system comprising:

(i) at least two hollow fiber bioreactors including nutrient permeable hollow fibers positioned in extracapillary space of said bioreactors;

(ii) a loop for controlling the flow of fluids through the lumens of said fibers, said loop comprising:

(a) pump means for circulating fluids through said loop;

(b) means for introducing gases into said loop;

(c) means for determining and controlling the temperature of said fluids;

(d) means for determining and controlling the dissolved oxygen content of said fluids upstream or downstream of said bioreactors;

(e) means for determining and controlling the flow rate of said fluids through said loop;

(f) means for determining and controlling pH of said fluids;

(g) means for introducing fluids comprising basal medium containing said nutrient into said loop; and

(iii) means for controlling the flow of fluids through said extracapillary spaces.

2. A hollow fiber cell propagation system as defined by claim 1 in which said means (iii) comprises means for providing a controlled fluid flow rate for introduction of fluids into and removal of product from said extracapillary spaces of each of said bioreactors, with fluid flow from said means (iii) and through each of said bioreactors controlled by valve means, wherein said valve means permits said fluid flow only through one of said bioreactors at a time.

3. A hollow fiber cell propagation system as defined by claim 1 in which said loop for controlling flow of fluids through said bioreactors is connected to each of said bioreactors through manifold means with flow from said loop provided into said manifold means diagonally opposite from flow out of said manifold means and said bioreactors being of the same internal volume with said bioreactors connected in parallel by said manifold means.

4. A hollow fiber cell propagation system as defined by claim 1 in which said means (ii)(g) provides for introduction of said basal medium and reagents into said loop at a rate determined by a preselected nutrient utilization rate in said bioreactors.

5. A hollow fiber cell propagation system as defined by claim 1 in which said means (iii) comprises means for providing a controlled fluid flow rate for simultaneous introduction of fluids into and removal of product from said extracapillary spaces.

6. A hollow fiber cell propagation system as defined by claim 1 in which said nutrient permeable hollow fibers are formed of a synthetic polymer.

7. A hollow fiber cell propagation system as defined by claim 6 in which said nutrient permeable hollow fibers are formed from a polymethylmethacrylate, a polysulfone or a cellulose derivative.

8. A hollow fiber cell propagation system as defined by claim 1 in which said means (ii)(c) for determining and controlling the temperature of said fluids comprises a resistance temperature probe and a resistance temperature probe flow block for insertion into said loop, said probe being connected to an electronic monitor and controller, said controller including means to compare the signal from said probe with a pres lected set point.

9. A hollow fiber cell propagation system as defined by claim 1 in which said means (ii)(c) for determining and controlling the temperature of said fluids comprises a resistance heated conduit for passage of said fluids.

10. A hollow fiber cell propagation system as defined by claim 9 in which said means (ii)(c) for determining and controlling the temperature of said fluids comprises an etched foil resistance heating element.

11. A hollow fiber cell propagation system as defined by claim 1 in which said loop (ii) includes means for determining the dissolved oxygen content of said fluids both upstream and downstream of said bioreactors.

12. A hollow fiber cell propagation system as defined by claim 1 further comprising means for determining the difference between the dissolved oxygen content of said fluids upstream and downstream of said bioreactors.

13. A hollow fiber cell propagation system as defined by claim 1 further comprising a means (iv) for determining the difference between the dissolved oxygen content of said fluids in said loop upstream and downstream of said bioreactors and for utilizing said difference to determine the aerobic efficiency and provide a real time verification and measurement of cell metabolism in said bioreactors.

14. A hollow fiber cell propagation system as defined by claim 13 further comprising means for utilizing the difference in the dissolved oxygen content of said fluids upstream and downstream of said bioreactors to assess the performance of said bior actors.

15. A hollow fiber cell propagation system as defined by claim 1 in which said means (e) for determining and controlling the introduction of basal medium through said loop comprises nutrient and ammonia analyzers.

16. A hollow fiber cell propagation system as defined by claim 15 further comprising means for utilization of data provided by said analyzers to provide for the introduction of basal medium into said loop at a preselected rate.

17. A hollow fiber cell propagation system as defined by claim 1 in which said means ii(b) for introducing gases into said loop further comprises an oxygenator means for introducing gases into basal medium after oxygen depletion due to passage through said bioreactors.

18. A hollow fiber cell propagation system as defined by claim 17 in which said oxygenator means includes means for blending air, nitrogen and carbon dioxide prior to introduction into said oxygen depleted basal medium.

19. A hollow fiber cell propagation system as defined by claim 17 in which said oxygenator means comprises a plurality of semipermeable hollow fibers membranes for passage of said oxygen depleted basal medium.

20. A hollow fiber cell propagation system as defined by claim 1 further comprising a waste disposal means of automatically displacing from said loop a volume substantially identical to the volume of fresh basal medium introduced into said loop.

21. A hollow fiber cell propagation system comprising:

(i) at least two hollow fiber bioreactors including nutrient permeable hollow fibers positioned in extracapillary space of said bioreactors;

(ii) a loop for controlling the flow of fluids through the lumens of said fibers, said loop comprising:

(a) pump means for circulating fluids through said loop;

(b) means for introducing gases into said loop;

(c) means for determining and controlling the temperature of said fluids;

(d) means for determining and controlling the dissolved oxygen content of said fluids upstream or downstream of said bioreactors;

(e) means for determining and controlling the flow rate of said fluids through said loop;

(f) means for determining and controlling pH of said fluids;

(g) means for introducing fluids comprising basal medium and reagents into said loop;

(h) means for providing for introduction of said basal medium and reagents into said loop;

(iii) means for controlling the flow of fluids through said extracapillary space; and

(iv) means for determining the difference between the dissolved oxygen content of said fluids in said loop upstream and downstream of said bioreactor and for utilizing said difference to determine the aerobic efficiency and provide a real time verification and measurement of cell metabolism in said bioreactors.

22. A hollow fiber cell propagation system as defined by claim 21 in which said means (iii) comprises means for providing a controlled fluid flow rate for introduction of fluids into and removal of product from said extracapillary spaces of each of said bioreactors, with fluid flow from said means (iii) and through each of said bioreactors controlled

by valve means, wherein said valve means permits said fluid flow only through one of said bioreactors at a time.

23. A hollow fiber cell propagation system as defined by claim 21 in which said loop for controlling flow of fluids through said bioreactors is connected to each of said bioreactors through manifold means with flow from said loop provided into said manifold means diagonally opposite from flow out of said manifold means and said bioreactors being of the same internal volume with said bioreactors connected in parallel by said manifold means.

24. A process for the conversion of a raw material to a useful product by cell metabolism which comprises

(i) inoculating the extracapillary space of at least two hollow fiber bioreactors with cells and a growth factor mixture for said cells;

(ii) circulating basal medium comprising a nutrient into and through the lumens of the hollow fibers of said bioreactors;

(iii) introducing oxygen into said circulating basal medium to establish and maintain an oxygen content sufficient to support aerobic metabolism of said cells;

(iv) utilizing the differential in dissolved oxygen content of the circulating basal medium before and after passage through the lumens of the hollow fibers of said bioreactors to provide a real time verification of cell metabolism.

25. A process as defined by claim 24 in which said inoculation of cells and growth factor mixture into said extracapillary space in step (i) comprises controlled fluid flow for introduction of fluids into

and removal of product from said extracapillary space of each of said bioreactors with said fluid flow permitted through only one of said bioreactors at a time.

26. A process as defined by claim 24 in which fluid flow of said circulatory basal medium in step (ii) comprises having said bioreactors being of the same internal volume and said bioreactors connected in parallel by use of manifold means with said fluid flow passing into said manifold means diagonally opposite from fluid flow out of said manifold means.

27. A process as defined by claim 24 in which oxygen is introduced into said circulating basal medium in step (iii) by passage of said medium through the lumens of a hollow fiber oxygenator in which gases containing oxygen are present in the extracapillary space surrounding said hollow fibers of said oxygenator.

28. A process as defined by claim 25 further comprising simultaneously introducing said growth factor into and removing product from said extracapillary space of said hollow fiber bioreactor at rates which do not differ by more than about five percent.

29. A process as defined by claim 24 in which said nutrient is maintained at a level at about one millimolar in said bioreactor.

30. A process as defined by claim 1 or claim 29 in which said nutrient is glucose.

31. A process as defined by claim 24 in which said conversion is accomplished by mammalian, insect, avian, plant, bacterial or fungal cells.

32. A process is defined by claim 24 in which said conversion is accomplished by anchorage dependent cells.

33. A process as defined by claim 24 in which said conversion in accomplished by suspension cells.

34. A process as defined by claim 24 in which said conversion is accomplished by cells which yield a virus or viral protein.

35. A process as defined by claim 33 in which said conversion is accomplished by a hybridoma.

36. A process as defined by claim 24 in which the rate of circulation of said basal medium into and through the lumens of said hollow fibers in said bioreactors is controlled by a predetermined bioreactor nutrient utilization rate and dissolved oxygen differential.

37. A process as defined by claim 36 in which said nutrient is glucose and in which the concentration of said glucose is maintained at about one millimolar in said bioreactor.

38. A process as defined by claim 24 in which the adverse effects of product and metabolite feedback inhibition are ameliorated by control of rate at which fluids are introduced into said extracapillary space and product is removed from said extracapillary space.

39. A process as defined by claim 24 in which the temperature in said bioreactor is maintained at about 37°C.

40. A process as defined by claim 24 in which samples from said circulating basal medium are subjected to ammonia and nutrient analyses and the data from such analyses is used to determine a rate of introduction of basal medium into said system.

41. A process as defined by claim 40 in which said nutrient is glucose and said data is used to determine the rate of introduction of basal medium required to provide a preselected glucose utilization rate.

42. A process as defined by claim 24 in which said conversion is accomplished by an anchorage dependent or suspension type mammalian cells.

43. A hollow fiber process for the conversion of a raw material by cell metabolism which comprises

(i) inoculating the extracapillary space of at least two hollow fiber bioreactors with cells and a growth factor mixture for said cells;

(ii) passing basal medium and nutrients from a storage vessel into and through the lumens of said hollow fibers;

the rate of passage of said medium and reagents through said lumens being controlled by a preselected bioreactor nutrient utilization rate;

(iii) introducing oxygen into said basal medium and nutrients in a predetermined amount sufficient to support aerobic metabolism of said cells;

(iv) determining the differential in the dissolved oxygen content of said basal medium and nutrients upstream and downstream of said bioreactors;

(v) utilizing said dissolved oxygen differential to determine the aerobic efficiency of the cell metabolism in said bioreactors and to provide a real time verification and measurement of said cell metabolism; and

(vi) simultaneously introducing growth factor mixture into and removing product from said extracapillary space at rates which do not differ by more than about 5%.

44. A process as defined by claim 43 in which said inoculation of cells and growth factor mixture into said extracapillary space in step (i) comprises controlled fluid flow for introduction of fluids into

and removal of product from said extracapillary space of each of said bioreactors with said fluid flow permitted through only one of said bioreactors at a time.

45. A process as defined by claim 43 in which fluid flow of said basal medium in step (ii) comprises having said bioreactors being of the same internal volume and said bioreactors connected in parallel by use of manifold means with said fluid flow passing into said manifold means diagonally opposite from fluid flow out of said manifold means.

46. A process as defined by claim 43 in which said conversion is accomplished by mammalian cells and in which the basal medium comprises glucose.

47. A process as defined by claim 43 in which said mammalian cells are anchorage dependent.

48. A process as defined by claim 43 in which said mammalian cells are of suspension type.

49. A hollow fiber cell propagation system as defined by claim 1 in which said loop (ii) comprises means for introducing said nutrient into said loop separately from said basal medium.

50. A hollow fiber cell propagation system as defined by claim 1 in which said means (ii)(d) for determining and controlling the dissolved oxygen content of said fluids comprises a dissolved oxygen probe positioned in a flow cell and a window in the wall of said flow cell through which said probe may be observed.

51. A hollow fiber cell propagation system defined by claim 1 further comprising means for a separating control of fluids in said loop from fluids in said extracapillary space.

52. A hollow fiber cell propagation system as defined by claim 50 further comprising means for on-line measurement of the dissolved oxygen content of said fluids.

53. A hollow fiber cell propagation system as defined by claim 1 further comprising means for determining the value of the expression $\frac{GUR}{\Delta DO}$ where GUR means glucose utilization rate and ΔDO means dissolved oxygen differential.

54. A hollow fiber cell propagation system defined by claim 53 further comprising means for using said $\frac{GUR}{\Delta DO}$ value to determine aerobic efficiency of said system.

55. A hollow fiber cell propagation system as defined by claim 13 or claim 53 further comprising means for utilizing the difference in dissolved oxygen content upstream and downstream of said bioreactors to assess the efficiency of production of cellular products in said bioreactors.

56. A hollow fiber cell propagation system as defined by claim 1 further comprising an oxygenator means having a plurality of permeable hollow fibers positioned in a extracapillary means for passing oxygen depleted medium through the lumens of said hollow fibers and means for passing air, oxygen and nitrogen or a blend thereof into and through said extracapillary means for introduction into oxygen depleted medium in the lumens of said hollow fibers.

57. A hollow fiber cell propagation system as defined by claim 56 further comprising means for controlling oxygen content of said medium passed through said extracapillary means.

58. A hollow fiber cell propagation system as defined by claim 56 or claim 57 further comprising means for controlling the amount of air, carbon

dioxid and nitrogen introduc d into said medium passing through the lumens of said hollow fibers of said oxygenator and thereby regulate the pH and oxygen content of the medium upon exit from said oxygenator.

59. A hollow fiber cell propagation system as defined by claim 56 further comprising a waste disposal system having a siphon break to preclude siphoning of said medium from the reservoir as a result of venting across the oxygenator hollow fibers.

60. A hollow fiber cell propagation system as defined by claim 1 or claim 50 further comprising a programmable controller means to control said means (iii) and said means for introducing said nutrient into said loop separately from said basal medium.

61. A process for conversion of a raw material into a useful product by cell metabolism which comprises:

(i) inoculating the extracapillary space of at least two hollow fiber bioreactors with cells and a growth factor for said cells;

(ii) circulating basal medium comprising a nutrient into and through the lumens of the hollow fibers of said bioreactors;

(iii) introducing oxygen into said circulating basal medium to establish and maintain an oxygen content sufficient to support aerobic metabolism of said cells; and

(iv) simultaneously measuring the glucose utilization rate (GUR) of said bioreactor and the dissolved oxygen (ΔDO) differential across said bioreactor and using the GUR/ΔDO ratio to assess efficiency of production of cellular products of said bioreactors.

62. A process as defined by claim 61 in which said GUR/ΔDO ratio is maintained at 3 to 6 (mg/hr) % air saturation based on a recirculation rate of about 350 ml/min per bioreactor.

63. A process as defined by claim 61 in which steps (ii) and (iii) are conducted in response to a programmable controller.

64. A process as defined by claim 62 in which feedback inhibition is precluded by a programmable controller.

65. A process for the conversion of a raw material into a useful product by cell metabolism which comprises:

(i) inoculating the extracapillary space of at least two hollow fiber bioreactors with cells and a growth factor for said cells;

(ii) introducing a glucose free basal medium into a means for circulating fluids through the lumens of the hollow fibers of said bioreactors;

(iii) separately introducing glucose into said means for circulating fluids through the lumens of said hollow fibers of said bioreactors;

(iv) introducing oxygen into said circulating basal medium to establish and maintain an oxygen content sufficient to support aerobic metabolism of said cells; and

(v) simultaneously measuring the glucose utilization rate (GUR) of said bioreactors and the dissolved oxygen (ΔDO) differential across said bioreactors and using the GUR/ΔDO ratio to assess the efficiency of production of cellular products in said bioreactors.

66. A process as defined by claim 65 further comprising:

(vi) monitoring and controlling one or more of steps (i) through (v) with a programmable controller.

67. A hollow fiber process for the conversion of a raw material by cell metabolism which comprises

(i) inoculating the extracapillary space of at least two hollow fiber bioreactors with cells and a growth factor for said cells;

(ii) passing basal medium and nutrients from a storage vessel into and through the lumens of said hollow fibers;

the rate of passage of said medium and reagents into said lumens being controlled by a preselected bioreactor glucose utilization rate;

(iii) introducing oxygen into said basal medium and nutrients in a predetermined amount sufficient to support aerobic metabolism of said cells;

(iv) simultaneously measuring the glucose utilization rate (GUR) of said bioreactors and the dissolved oxygen (ΔDO) differential across said bioreactors and using the GUR/ΔDO ratio to assess the efficiency of production of cellular products in said bioreactors; and

(v) monitoring and controlling one or more of steps (i) through (iv) with a programmable controller.

68. A hollow fiber cell propagation system as defined by claim 50 in which said dissolved oxygen probe is calibrated by sterile introduction of air through a filter means so said air is transported adjacent a membrane means on said dissolved oxygen probe, upon completion of calibration of said dissolved oxygen probe said air is removed through said filter m ans, while maintaining fluid flow

through said flow cell and having the pressure of said air adjacent said membrane means be the same as said fluid flowing through said flow cell.

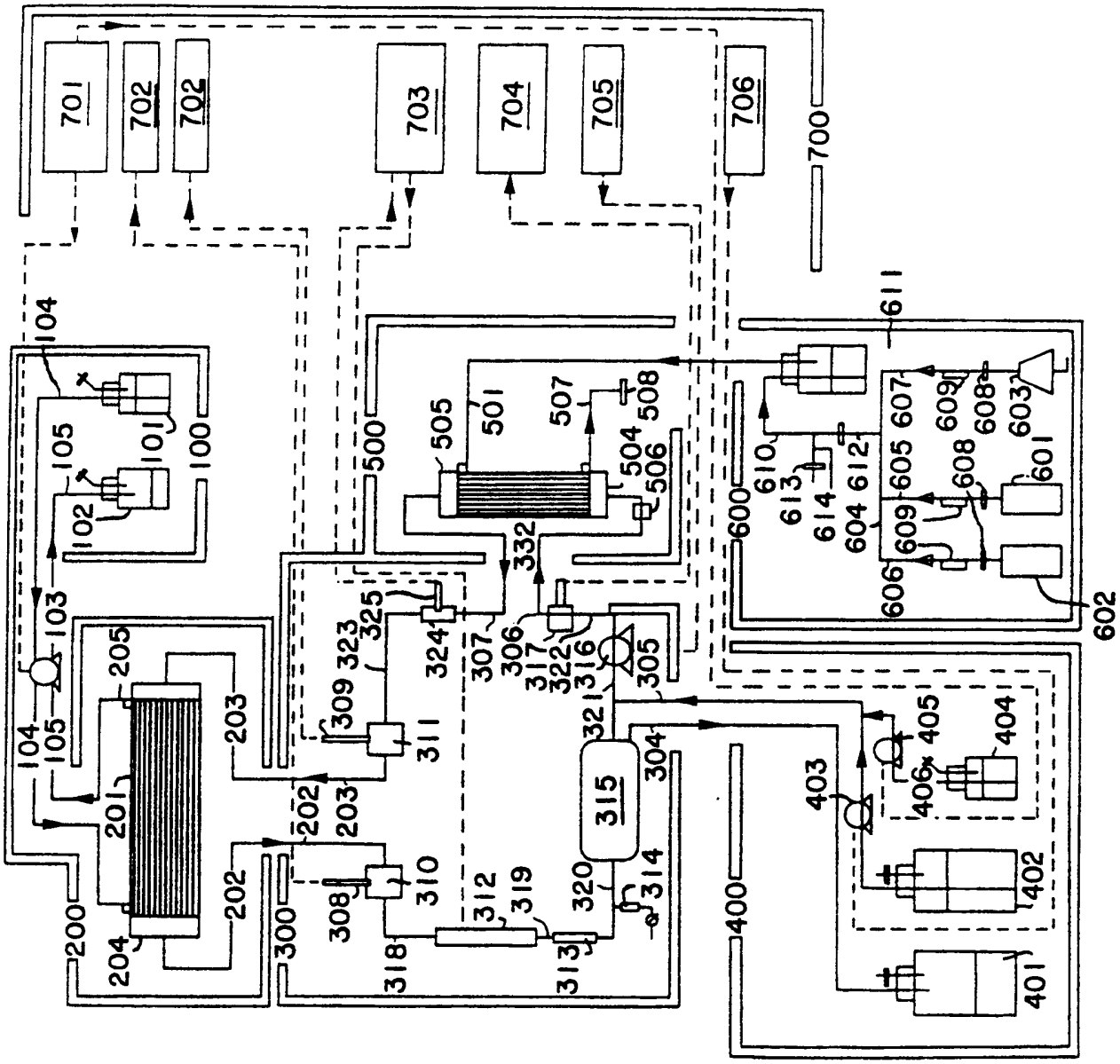
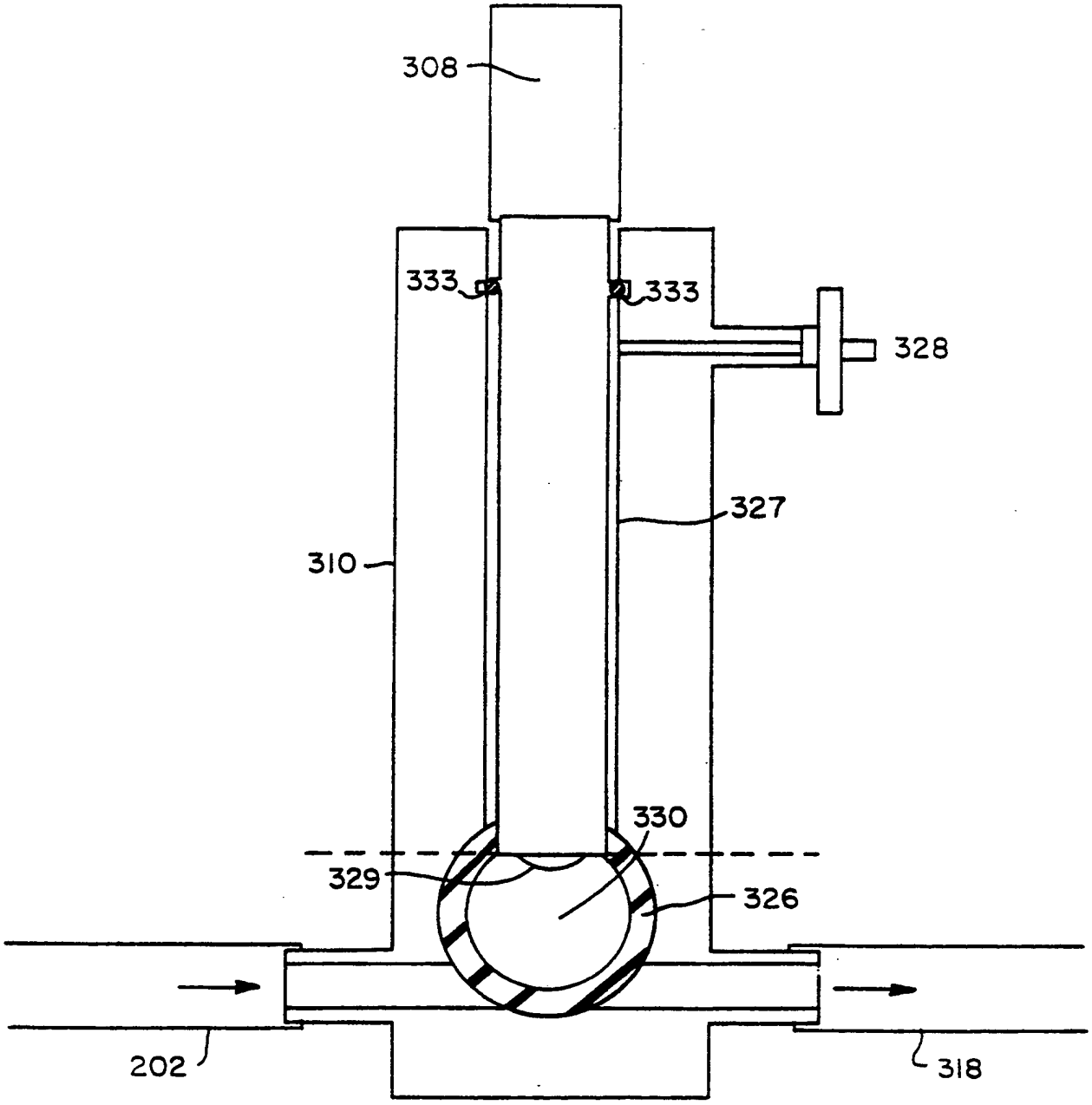


FIG. 1

FIG. 2



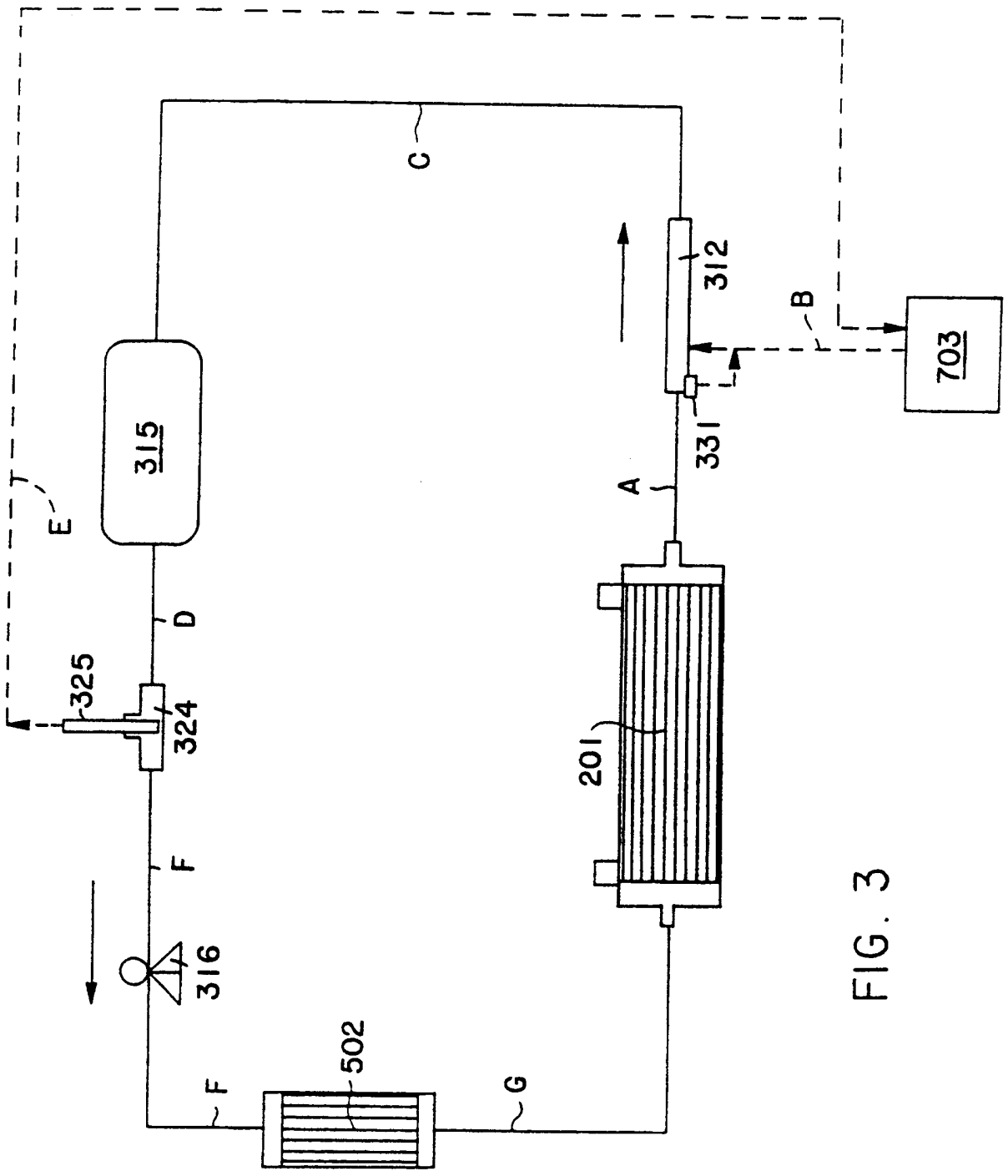


FIG. 3

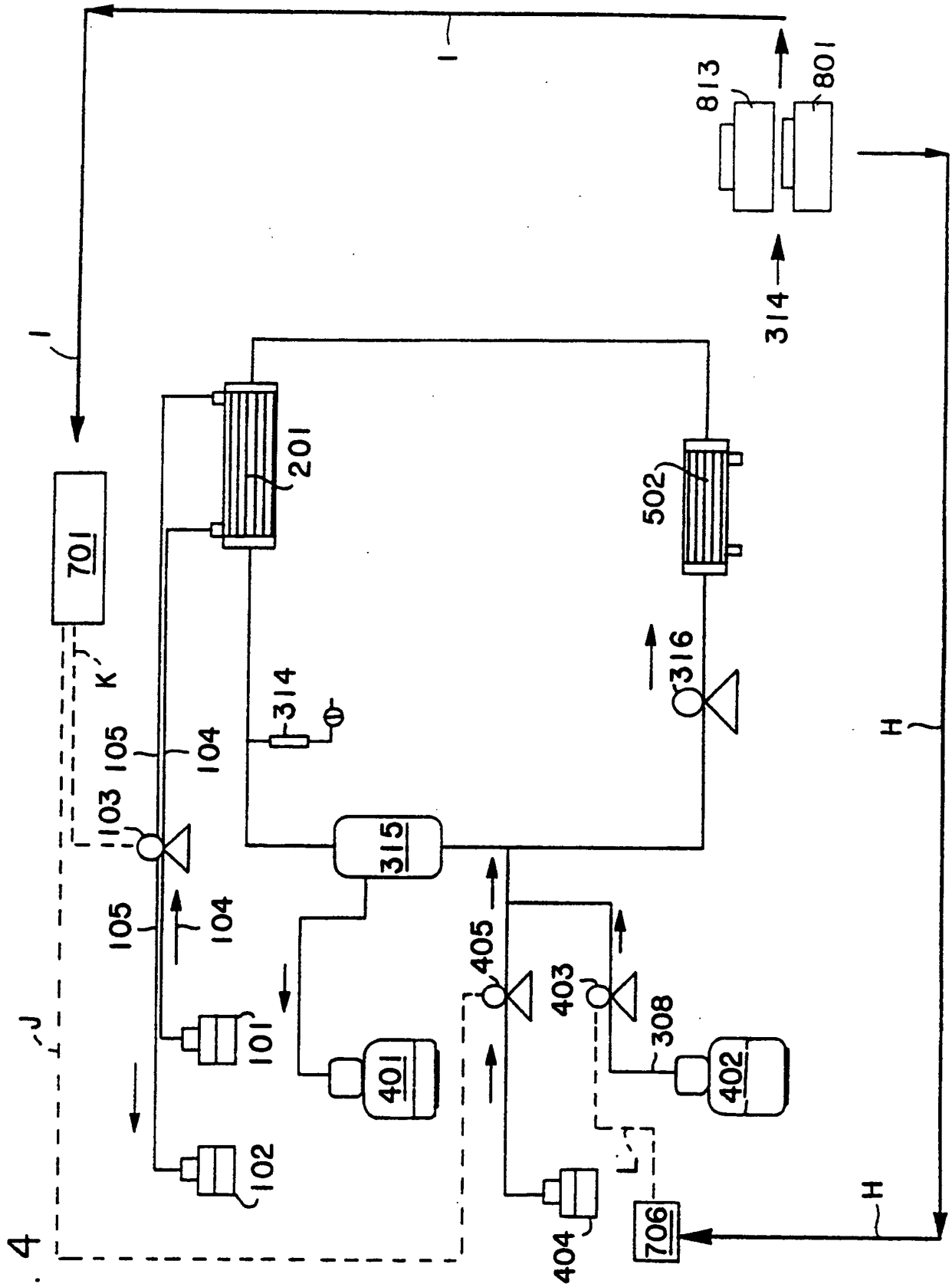

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FIG. 4

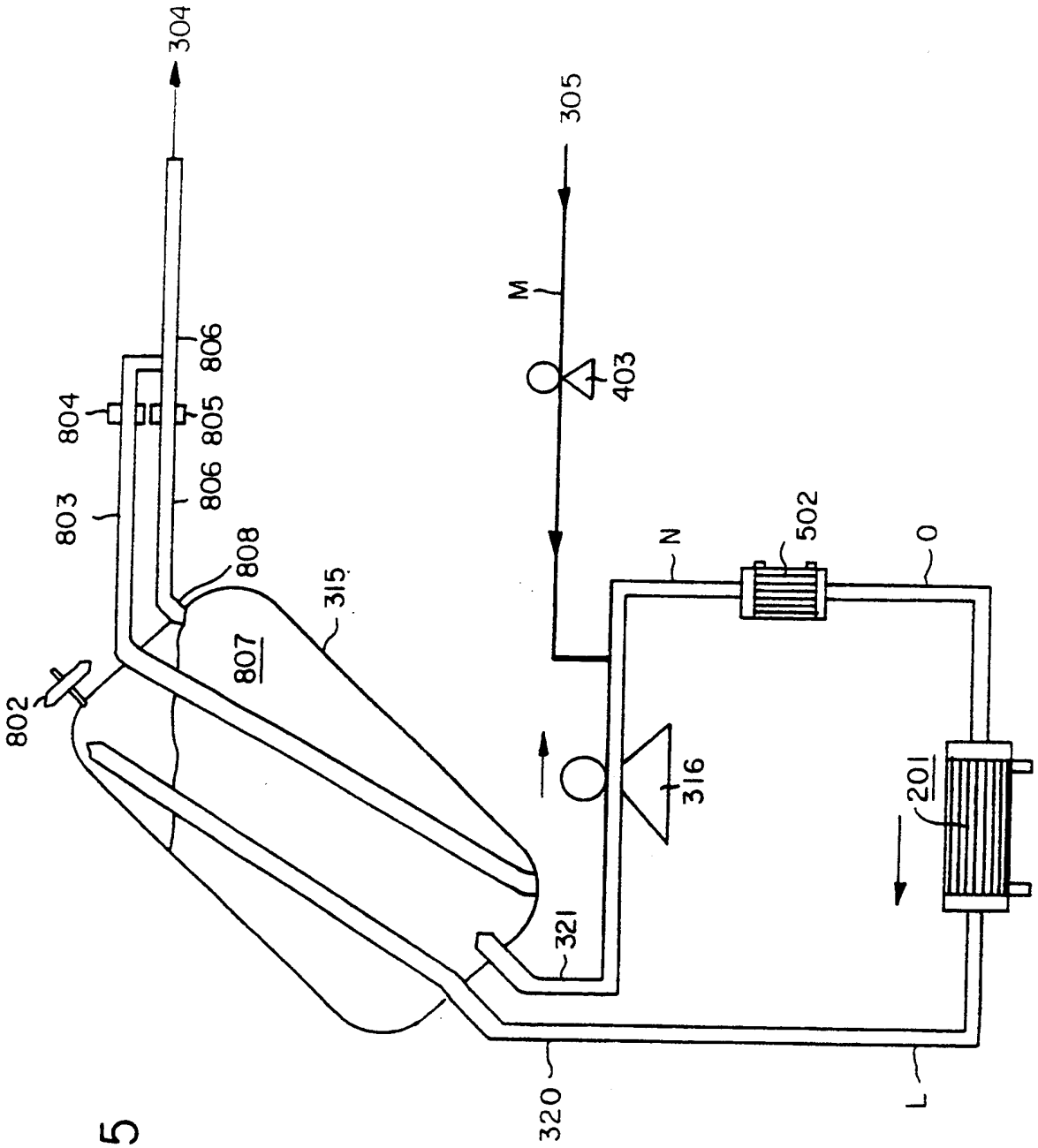
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FIG. 5

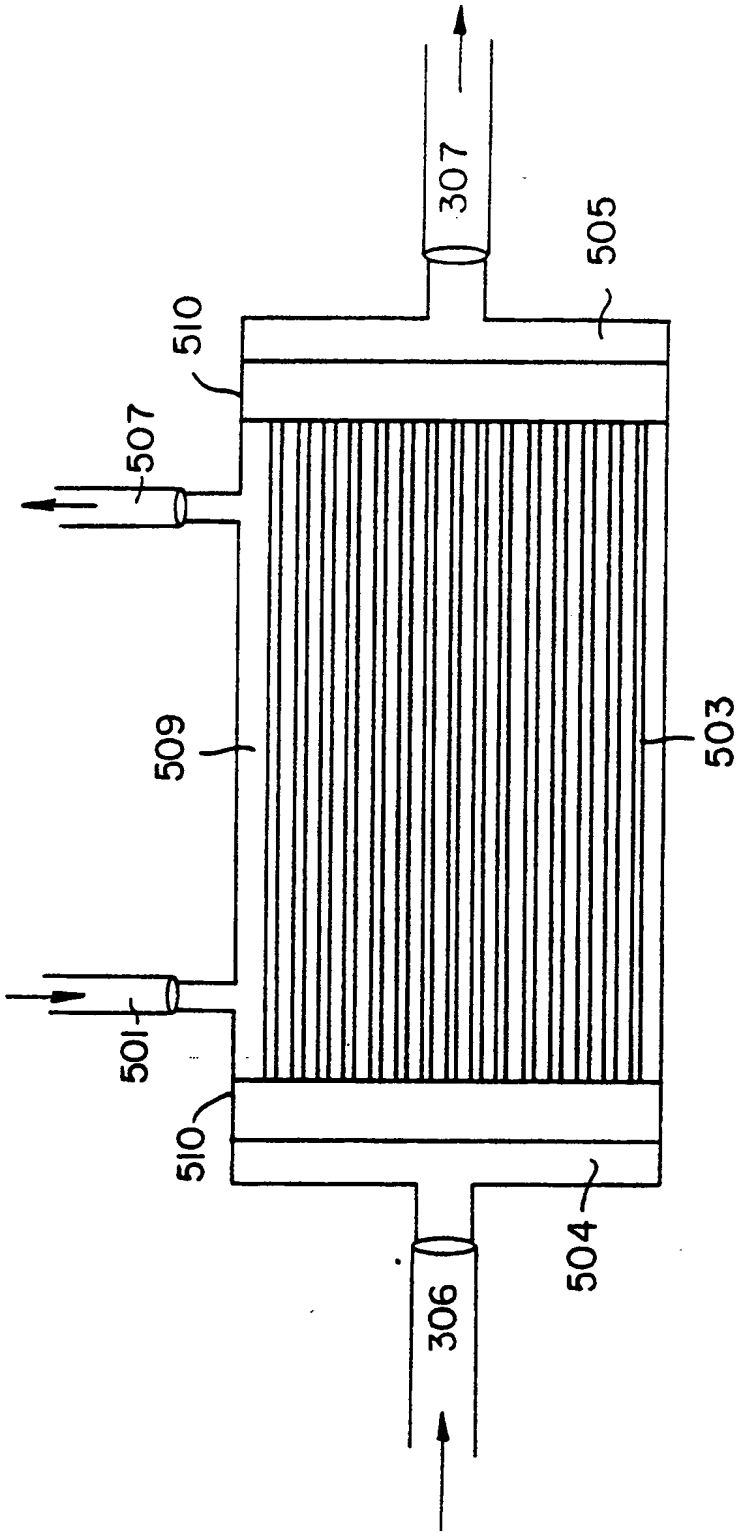
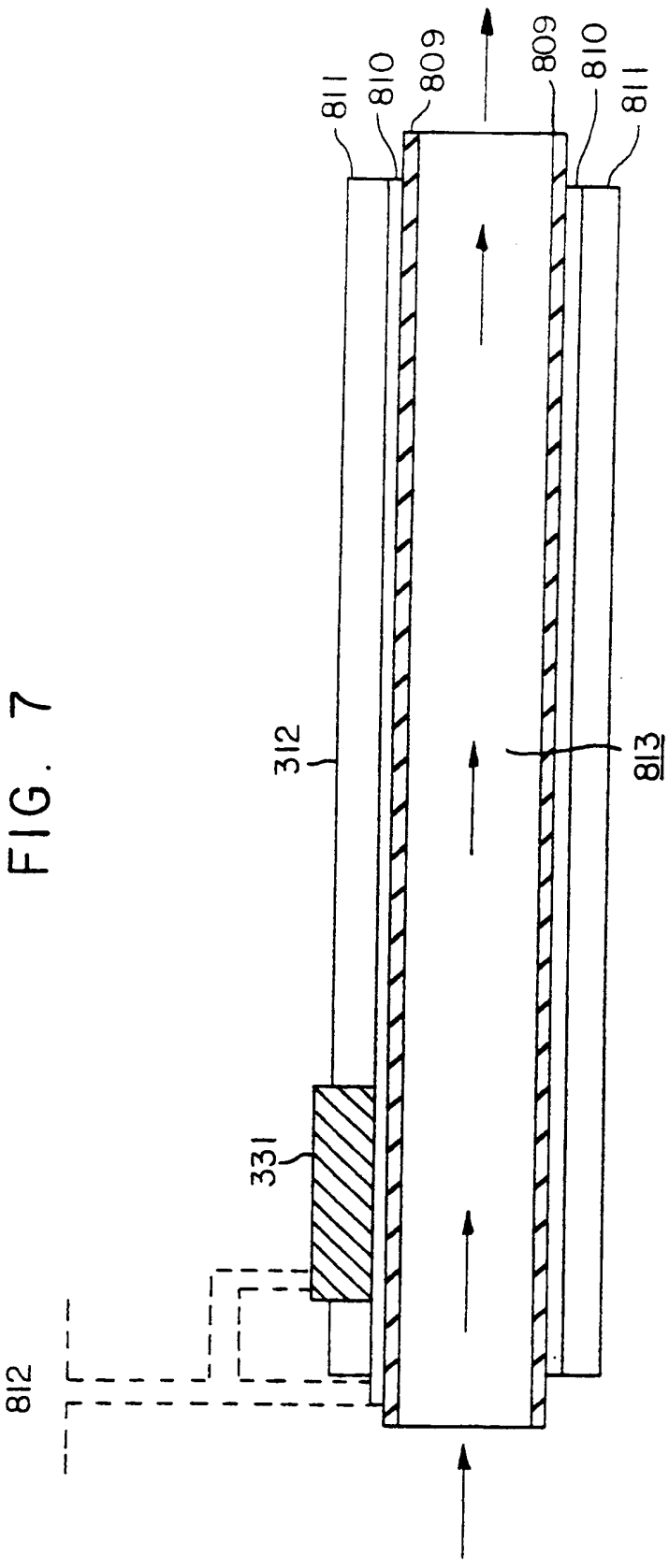


FIG. 6

FIG. 7



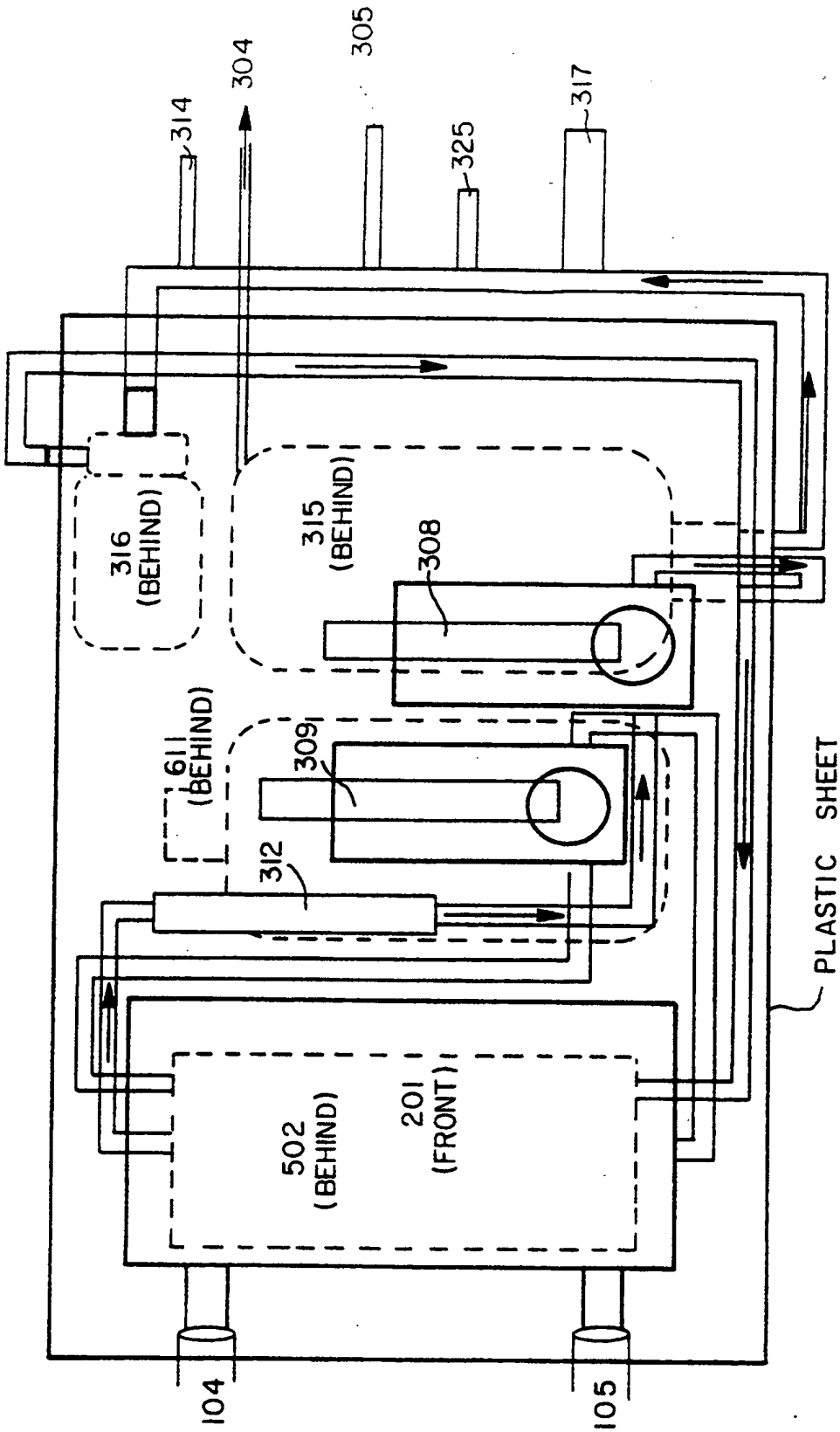

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FIG. 8

FIG. 9

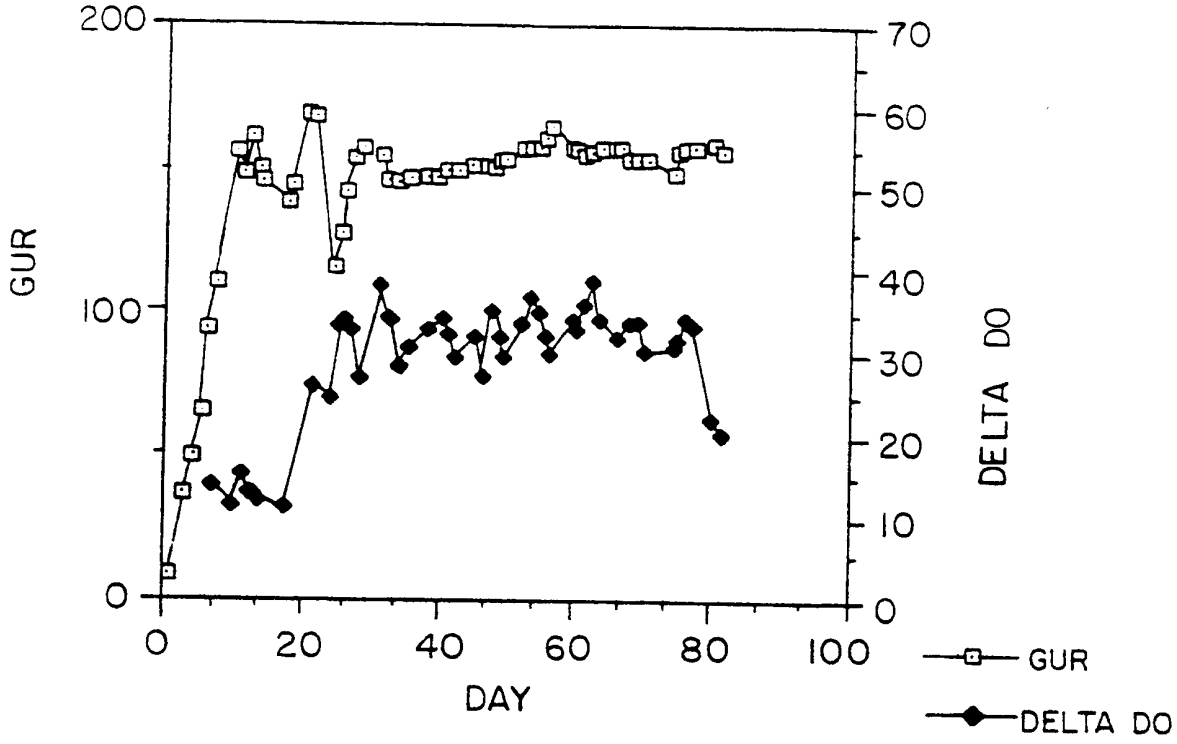
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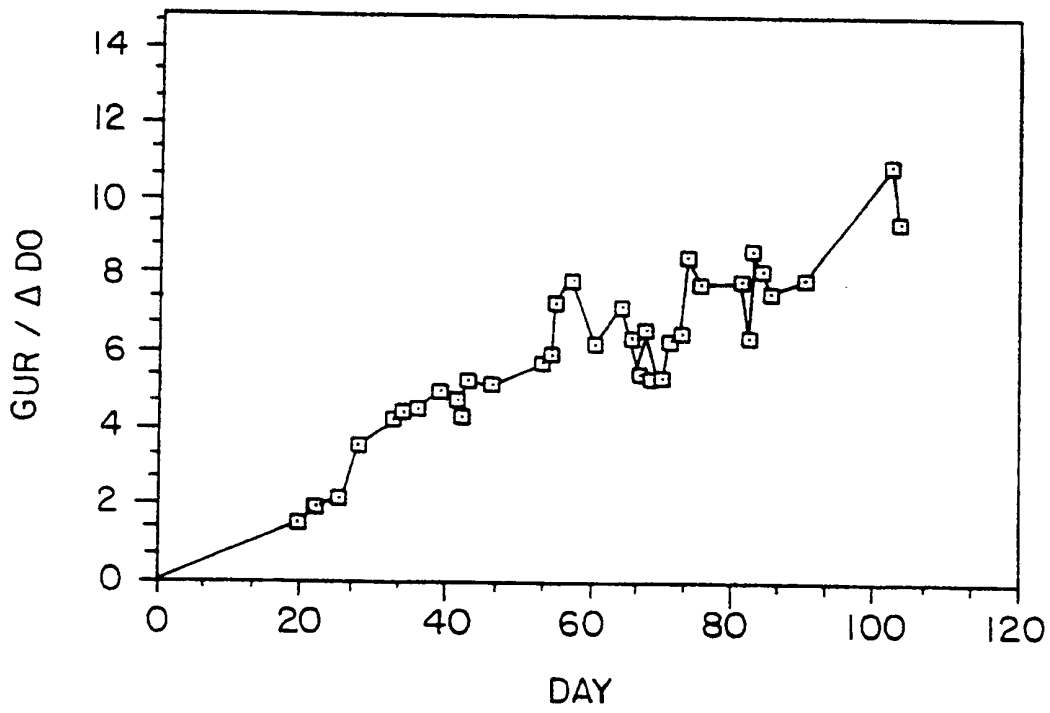
FIG. 10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GUR / Δ DO
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DAY

FIG. 11

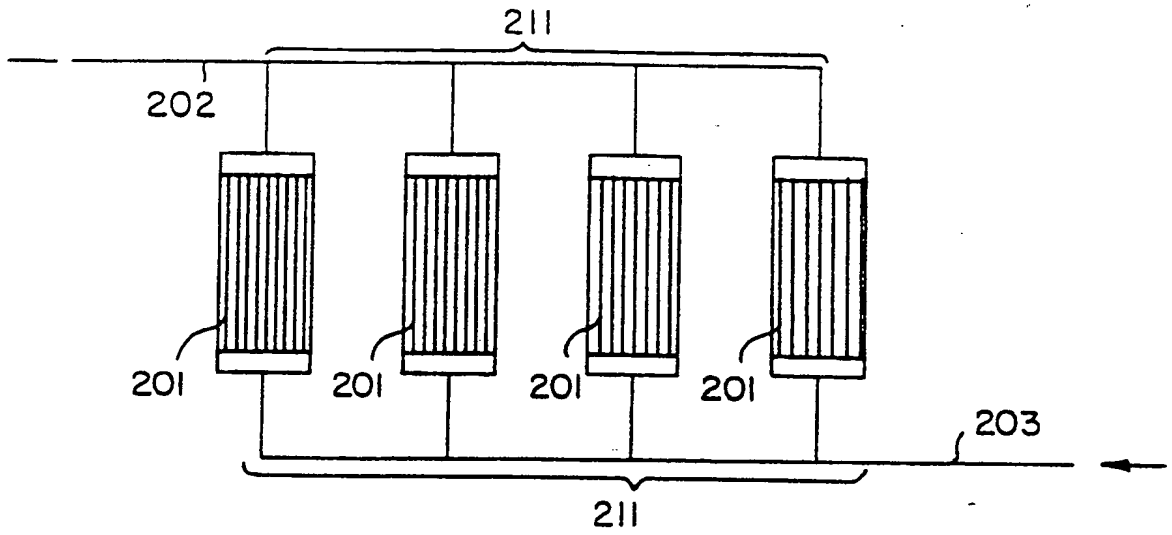
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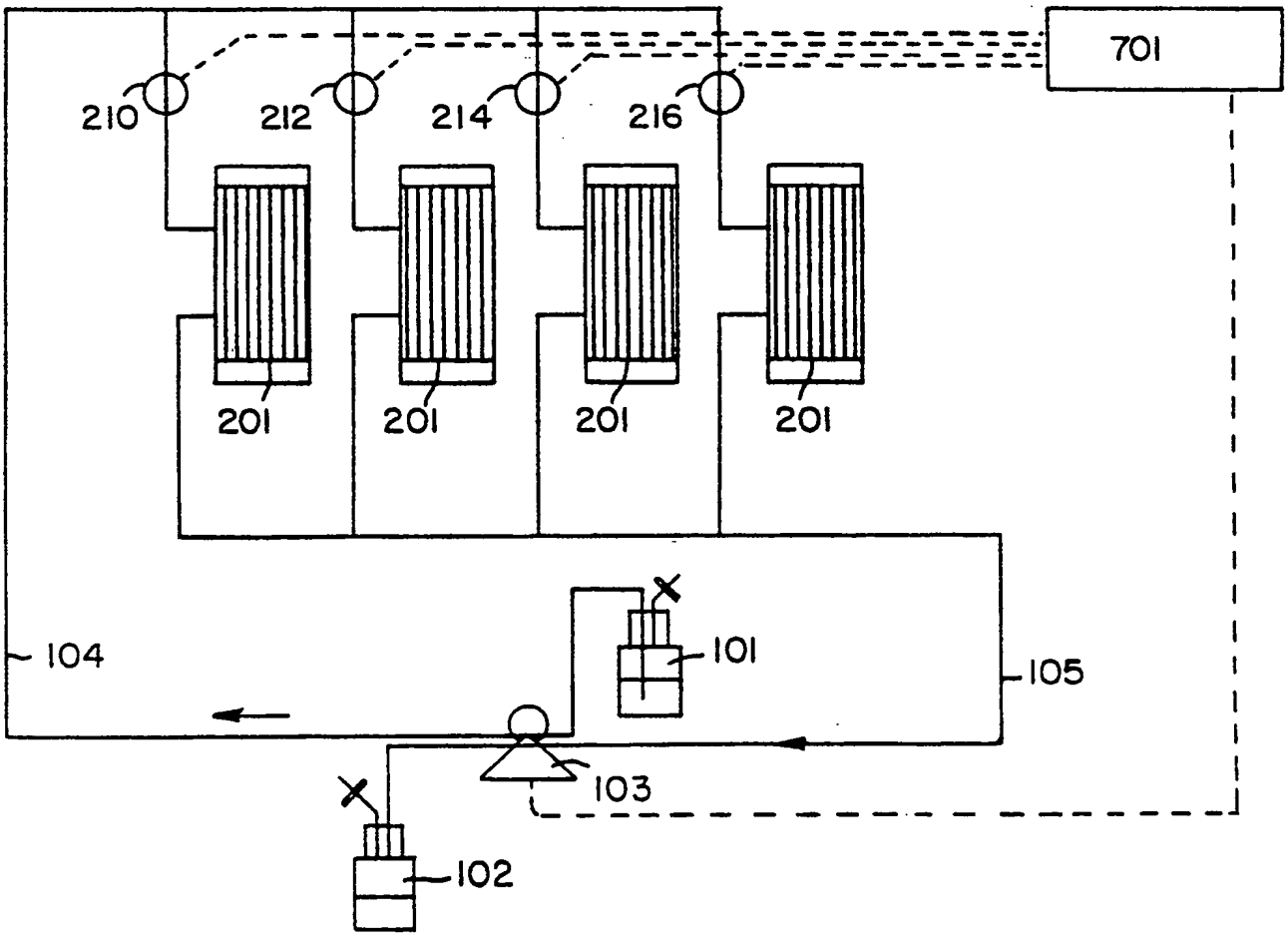
FIG. 12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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/01386

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12M 1/04, 1/36

U.S. Cl.: 435/288, 289, 290, 291, 313, 807, 813

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. Cl.	435/288, 289, 290, 291, 313, 807, 813

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,764,471 (RIPKA) 16 August 1988, See entire document.	1-68
Y	US, A, 4,889,812 (GUINN, et al.) 26 December 1989, See entire document.	1-68
T	US, A, 4,999,298 (WOLFE, et al.) 12 March 1991, See entire document.	1-68
A	US, A, 4,720,462 (ROSENSON) 19 January 1988, See entire document	1-68
A	US, A, 4,220,725 (KNAZEK, et al.) 02 September 1980, See entire document.	1-68
Y	JP, A, 6,188,872 (KUMAZAWA) 07 May 1986, See Figure 1 and Abstract.	1-68

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12 April 1991	03 JUN 1991
International Searching Authority: ISA/US	Signature of Authorized Officer: Janelle D. Waack

Form PCT/ISA/210 (second sheet) (rev. 11-87)